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13. ABSTRACT (Maximum 200 Words) Cyclooxygenase-2 (COX-2) is an inducible enzyme which catalyzes the conversion of arachidonic acid to prostaglandins and has previously been demonstrated to play a role in carcinogenesis. We demonstrated that COX-2 and one of its major prostaglandin products, PGE ₂ , are mediators of hypoxia-induced increases in a potent angiogenic factor, VEGF, in a human prostate cancer cell line. In these studies we are determining (1) the optimal dosing and timing of a COX-2 inhibitor (NS398) in an animal model of human prostate cancer, (2) and (3) the mechanisms underlying the observed effects of COX-2 and PGE ₂ on hypoxia-induced upregulation of VEGF and tumor angiogenesis. Over the past year we have determined the optimal dose of NS398 in the in vivo model. We have also demonstrated that true hypoxia (as opposed to cobalt-chloride simulated hypoxia) induces VEGF expression in PC-3 ML prostate cancer cells and that NS398 prevents this effect while PGE ₂ restores it. Our studies demonstrate that PGE ₂ does increase the transcription of hypoxia-inducible factor-1 α (HIF-1 α) but does increase HIF-1 α protein expression, primarily in the cytoplasm. In the next two years, we will determine the optimal timing of NS398 administration and determine how PGE ₂ increases HIF-1 α protein and VEGF expression.				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5-9
Key Research Accomplishments.....	10
Reportable Outcomes.....	11
Conclusions.....	12
References.....	13-14
Appendices.....	15-
	(2 Reprints/13 pages total)

INTRODUCTION

Cyclooxygenase-2 (COX-2) is an inducible enzyme which catalyzes the conversion of arachidonic acid to prostaglandins. COX-2 plays a key role in cancer (1-4) and COX-2 inhibition prevents and treats colon cancer (5-7). Both COX-2 and its major derived prostaglandin product (PGE₂) have been implicated as stimulators of tumor angiogenesis (8-13).

We previously demonstrated that COX-2 expression is increased in human prostate cancer tissues (14) and that a COX-2 inhibitor selectively induces apoptosis in a prostate cancer cell line (15). We also demonstrated that treatment of human prostate tumor-bearing mice with a selective COX-2 inhibitor (NS-398) significantly reduces tumor size, microvessel density and levels of a potent tumor angiogenic factor, vascular endothelial growth factor (VEGF) (16) (APPENDIX, Manuscript #1). Our in vitro studies with a highly invasive human prostate cancer cell line, PC-3ML, demonstrated that cobalt-chloride simulated hypoxia induced VEGF upregulation. In that same report, VEGF upregulation by cobalt chloride simulated hypoxia was prevented by NS-398 treatment and restored by the addition of PGE₂ (one of the major prostaglandin products of the reaction catalyzed by COX-2) (17) (APPENDIX, Manuscript #2).

Based on our preliminary data, we hypothesized that COX-2 and the resultant PGE₂ are mediators of hypoxia-induced effects on VEGF in prostate cancer cells. We further hypothesized that PGE₂ mediates hypoxic upregulation of VEGF by modulating hypoxia-inducible factor-1 α (HIF-1 α), the major transcriptional regulator of VEGF expression. The first task of these studies was to determine the optimal dosing and timing of administration of NS-398 (COX-2 inhibitor) in prostate-tumor bearing mice. The second task was to determine the mechanisms underlying the observed effects of the COX-2 inhibitor and PGE₂ on hypoxia-induced upregulation of VEGF. Specifically, we proposed to determine whether PGE₂ itself regulates HIF-1 α and VEGF expression levels. Finally, in Task 3, we will determine whether PGE₂ modulates VEGF transcription by enhancing the binding of HIF to the promoter region of the VEGF gene.

BODY

Task 1. Perform studies using a mouse model of human prostate cancer to determine the *in vivo* efficacy of a selective COX-2 inhibitor as anti-tumor and anti-angiogenic agent.

A. Phase I Study: Optimal Dose of NS398 (COX-2 Inhibitor) In Vivo.

The *in vivo* efficacy of NS398 was examined in nude mice. Mice were randomly divided into 4 groups with 20 mice each and treated with: Group 1, vehicle only as control, Group 2, NS398 (0.5 mg/kg body weight), Group 3, NS398 (1.0 mg/kg body weight), and Group 4, NS398 (3.0 mg/kg body weight). As shown in Fig. 1, NS398, at all three doses, produced a suppression of PC-3ML tumor growth within 10 weeks after tumor cell inoculation. However, the mice treated with the low (0.5 mg/kg) and medium (1.0 mg/kg) doses of NS398 exhibited an increase in tumor growth after 10 weeks, in spite of continuous drug administration. These data indicate that the tumors become resistant to the two lower doses after 10 weeks. In contrast, mice treated with the highest dose (3 mg/kg) of NS398 exhibited a constant inhibition of tumor growth over a 15-week experimental period. Average tumor surface areas at week 10 were: group 1 (control) = 207.2 mm², group 2 (low dose NS-398) = 73.8 mm² (66.7 % inhibition of the control), group 3 (medium dose NS-398) = 45.6 mm² (72.3 % inhibition), and group 4 (highest dose NS-398) = 37.7 mm² (86.4 % inhibition). In contrast, average tumor surface areas at week 15 were 170.5 mm², 152.5mm², 159.1mm², and 75.1mm², in Groups 1-4, respectively. Complete regression of existing tumors was observed in some animals in all three groups treated with NS398. Six tumors of twenty (30 %) in group 4 (high dose), five tumors of twenty (25 %) in group 3 (medium dose), and 1 in group 2 (low dose) were not palpable at week 15 after drug treatment. There was no evidence of drug related toxicity in any of the mice.

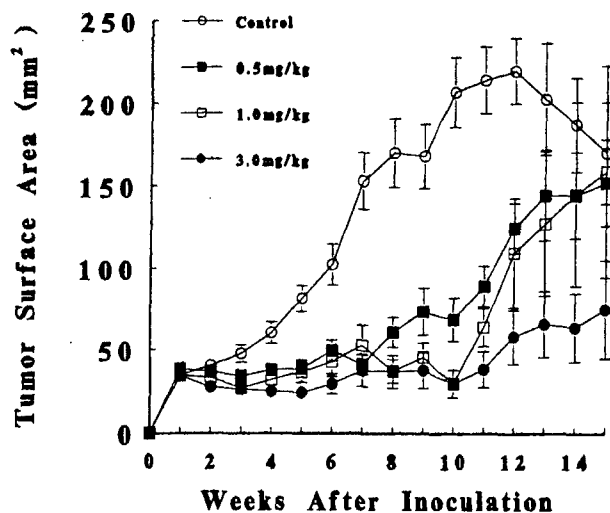


Fig. 1 NS398 induced a dose-dependent inhibition of prostate tumor growth in nude mice. From second week of PC-3ML cell inoculation, tumor-bearing mice were randomized to receive i.p. injections of either vehicle or one of the three doses (0.5, 1.0, and 3.0 mg/kg body weight) of a COX-2 inhibitor (NS-98), administered three times weekly. Tumor surface areas were measured weekly and expressed as means \pm SE in 20 tumors from each group.

These results confirm our previous findings (16) (APPENDIX, Manuscript #1) that selective COX-2 inhibition with NS-398 results in a potent inhibition of prostate tumor growth with low toxicity in mice. However, the dose-response studies demonstrate that only the highest dose (3 mg/kg/body weight) resulted in a sustained inhibition of tumor growth. Ongoing studies will determine the optimal timing of drug therapy (Task 1 [B]in original Statement of Work). In addition, we will try to subculture resistant tumors and determine the molecular markers associated with drug resistance.

Task 2: Determine the time course of hypoxic effects on VEGF and COX-2 expression and delineate the level at which NS398 (COX-2 Inhibitor) and prostaglandins regulate hypoxia-driven VEGF expression.

We recently reported on the effects of cobalt-chloride-simulated hypoxia on VEGF expression in PC-3 ML cancer cells. Cobalt chloride mimics some, but not all effects, of true hypoxia. We demonstrated that cobalt chloride greatly induced VEGF mRNA and protein in these cells and that this effect could be blocked by the addition of a COX-2 inhibitor (NS398) and restored by the addition of PGE₂ (a major COX-2 derived product) (17) (APPENDIX, Manuscript #2). In experiments conducted over the past year, we have demonstrated that true hypoxia (as opposed to cobalt-chloride simulated hypoxia) has the same effect on VEGF expression in these cells and that NS-398 can inhibit the effect of true hypoxia

Hypoxia stimulates VEGF secretion in PC-3ML cells.

Vascular endothelial growth factor (VEGF) is a potent tumor permeability and angiogenic factor (18-20). We have previously demonstrated that VEGF is critical to prostate cancer growth (21). One of the most potent VEGF stimulators is hypoxia which acts through hypoxia-inducible factor 1 α (HIF-1 α), an inducible transcription factor (22,23). We tested the effect of true hypoxia on the induction of VEGF protein secretion in PC-3ML cells. As shown in Fig. 2, treating tumor cells with hypoxia (1% Oxygen) significantly increased VEGF protein secretion in a time-dependent fashion.

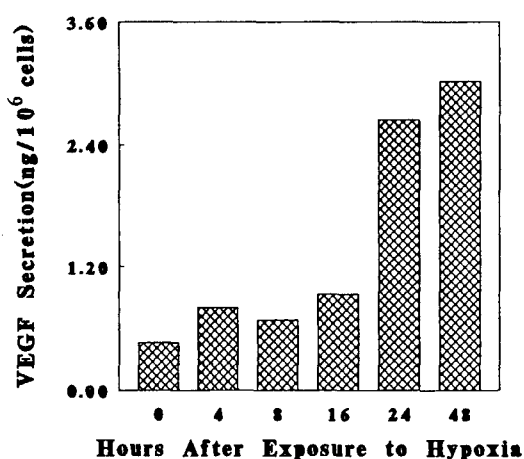


Fig. 2. Hypoxia stimulates VEGF secretion in PC-3ML cells. PC-3ML cells were incubated in serum-free medium supplemented with 0.1% BSA, and treated with either vehicle or 1% O₂ for the time points indicated. At the end of each time point, the medium was collected, clarified, and subjected to ELISA to quantitate VEGF protein secretion. The results were normalized to cell number.

NS398 inhibits hypoxia-induced VEGF secretion by PC-3ML cells.

We have reported that NS398, a selective COX-2 inhibitor, can prevent VEGF upregulation in response to cobalt-chloride induced hypoxia (17) (APPENDIX, Manuscript #2). In these studies we determined whether NS398 can also inhibit VEGF upregulation induced by true

hypoxia.. As shown in Fig. 3, NS398 has no observed effect on VEGF production under normoxic conditions. In contrast, NS398 significantly suppressed VEGF secretion induced by hypoxia in a dose- and time-dependent fashion.

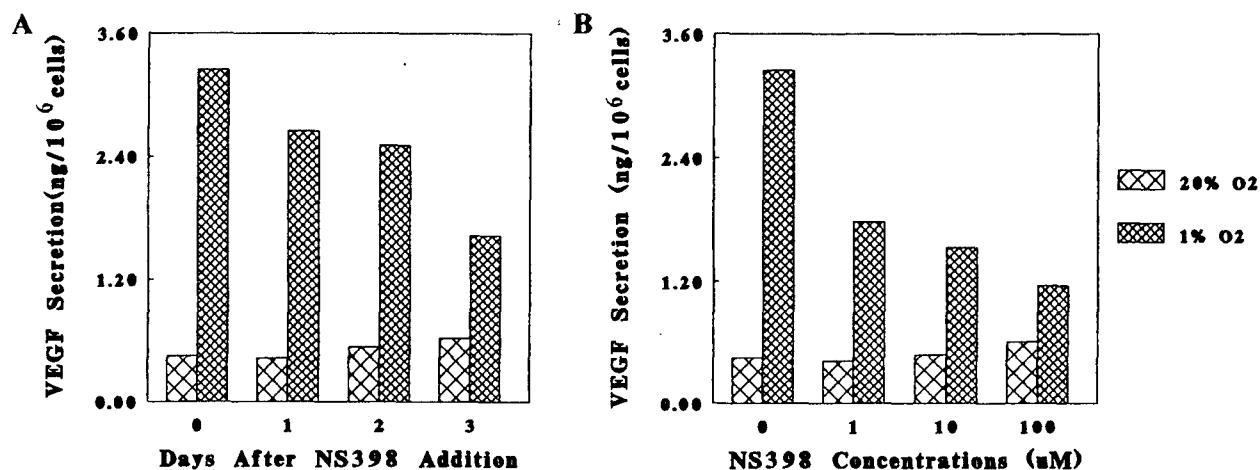


Fig. 3. NS398 inhibits hypoxia-induced VEGF secretion by PC-3ML cells.

(A) Time-dependent inhibition of VEGF secretion by NS398 in hypoxic PC-3ML cells. Cells were incubated in serum-free medium under hypoxic (1% O₂) or normoxic (20% O₂) conditions in the absence or presence of 100 μM NS398 for the time points indicated. **(B) Dose-dependent inhibition of hypoxia-induced VEGF secretion by NS398.** Cells were incubated in serum-free medium under hypoxic (1% O₂) or normoxic (20% O₂) conditions in the presence or absence of varying doses of NS398 for 2 d. Culture medium was then collected, clarified, and subjected to ELISA to quantitate VEGF secretion. The results were normalized to cell number.

Effects of PGE₂ on HIF mRNA expression in PC-3 ML cells

Previous studies have demonstrated that hypoxic upregulation of VEGF mRNA expression is mediated by a hypoxia-inducible transcription factor, HIF-1α (22,23). We hypothesized that the COX-2/prostaglandin pathway might be involved in the regulation of expression of HIF-1. Our data, both published (17) and from preliminary experiments (as shown above), suggest that PGE₂ plays a role in the process of hypoxia-induced VEGF expression. However, PGE₂ itself has only a minimum direct effect on VEGF induction (24). We hypothesized that PGE₂ may directly induce HIF-1α mRNA and/or protein expression under hypoxic conditions. Alternatively, PGE₂ may function as a co-factor which enhances the binding of HIF-1α protein to the promoter region of the VEGF gene, thereby enhancing hypoxia-driven upregulation of VEGF transcription.

To test these hypotheses, we initially examined the effects of PGE₂ on HIF-1 α mRNA expression. Rt-PCR, as shown in Fig. 4, revealed that PC-3ML cells express high basal levels of HIF-1 α mRNA and the levels did not change in response to PGE₂ addition (Fig. 4)

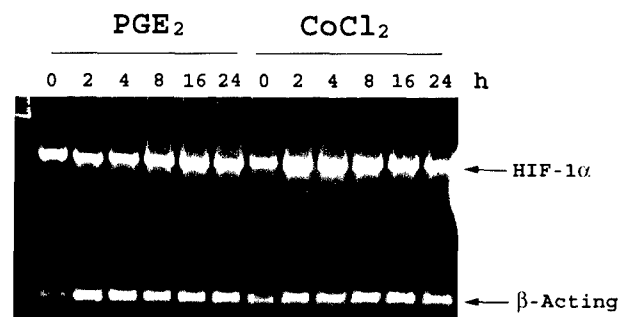


Fig. 4. Effects of PGE₂ on the expression of HIF-1 α mRNA in PC-3ML cells. PC-3ML cells were incubated in serum-free medium and treated with either vehicle or 1 μ M PGE₂ for various time, as indicated. At the end of each time point, total RNA was extracted with Trizol Reagent (Gibco-BRL, Gaithersburg, MD) according to the protocol from the supplier. cDNA was prepared by incubating 1 μ g of RNA in 50mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT and RNase inhibitors with 250 units of reverse transcriptase, 1 mM of each dNTP and random primers (0.05 mM, Gibco-BRL) for 60 min at 37°C. PCR was carried out using AmpliTaq DNA polymerase. Primer sequences were as following: sense (bp184-207), 5'-CGG-CGC-GAA-CGA-CAA-GAA-AAA-GAT-3' and antisense (bp 1327-1350), 5'-TCG-TTG-GGT-GAG-GGG-AGC-ATT-ACA-3'. PCR was initiated in a thermal cycle programmed at 95°C for 5 min, 94°C for 30 sec, 58°C for 30 sec, 72°C for 45 sec (28 cycles). A separate set of samples were obtained from the cells treated with cobalt chloride (CoCl₂) with same time points as control.

Effect of PGE₂ on HIF-1 α protein expression.

HIF-1 α protein has been thought to be a cytosolic protein which, upon stimulation by various factors, undergoes nuclear translocation. We examined the effect of PGE₂ on the expression and subcellular localization of HIF-1 α protein. Western blot analysis, as shown in Fig. 5, demonstrated that PGE₂ upregulates HIF-1 α protein expression with a peak induction at 8h after treatment. The induction mainly occurred in the cytosolic fraction.

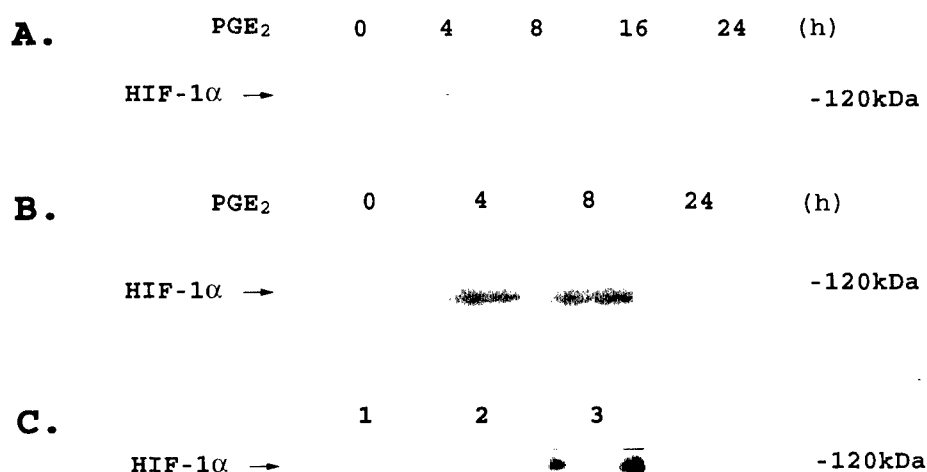


Fig. 5. PGE₂ upregulates HIF-1 α protein expression in the cytosolic fraction of PC-3ML cells. PC-3ML cells were cultured in serum-free medium and treated with either vehicle or 1 μ M PGE₂. Proteins were isolated and HIF-1 α protein expression was determined in (A) *Total Cell Lysates* (B) *Cytosolic Fraction* and (C) *Nuclear Fraction*, at the time points indicated (*h*=hours). Lane 1, cells treated with vehicle only as negative control; lane 2, cells treated with 1 μ M PGE₂ for 8h; lane 3, cells treated with 100 μ M CoCl₂ for 8h as positive control. The proteins were subjected to Western blot analysis. A representative result from duplicate experiments is shown.

KEY RESEARCH ACCOMPLISHMENTS

Over the past year (1st year of funding) we have:

- Established the optimal dose of a selective COX-2 inhibitor (NS398) which results in the maximal, sustained inhibition of prostate tumor growth in nude mice (3 mg/kg given intraperitoneally twice weekly).
- Demonstrated that true hypoxia upregulates VEGF expression in PC-3ML human prostate cancer cells and that NS398 (COX-2 inhibition) blocks this upregulation in a time- and dose-dependent fashion.
- Demonstrated that the major COX-2 derived prostaglandin product (PGE₂) has no effect on the mRNA expression of hypoxia-inducible factor-1 α (HIF-1 α) in PC-3 ML cells.
- Demonstrated that PGE₂ does upregulate HIF-1 α protein expression (in the absence of an effect on the mRNA levels) in PC-3 ML cells. This effect is primarily due to increased levels of HIF-1 α in the cytosolic fraction.

REPORTABLE OUTCOMES

We have not yet reported on the data from our first funded year. However, we have two very relevant publications (APPENDIX, MANUSCRIPTS #1 and #2) which were published within the last year and formed the basis for the present studies.

CONCLUSIONS

Task I: In Vivo Studies with NS398 (COX-2 Inhibitor)

We have demonstrated that the highest dose of COX-2 inhibitor (NS398, 3mg/kg given intraperitoneally twice weekly for 15 weeks) is the most efficacious in terms of a sustained reduction in prostate tumor volume. The two lower doses initially decreased tumor growth rates, but, after 10 weeks treatment, tumor growth rates increased. In the next phase of study, we will utilize this optimal dose and determine the optimal time of administration of the inhibitor (early vs. late) for maximal, sustained effects on tumor growth.

Task II: Mechanism of COX-2/PGE₂ mediated effects on VEGF and HIF-1 α in the Presence of True Hypoxia

These studies proved that true hypoxia increases VEGF expression in human prostate cancer cells and that this effect is mediated by COX-2.

Based upon our studies with cobalt chloride-simulated hypoxia (Manuscript #2, APPENDIX) we hypothesized that the major prostaglandin product of the COX-2 catalyzed reaction, PGE₂, is a mediator of hypoxia-induced VEGF regulation. We further hypothesized that PGE₂ increases the mRNA and/or protein expression of the major hypoxia-inducible transcription factor, HIF-1 α . The studies conducted over this initial year of funding have demonstrated that PGE₂ does not increase HIF-1 α mRNA levels. However, we have demonstrated an effect of PGE₂ on HIF-1 α protein levels, particularly in the cytoplasm.

The observed effects of PGE₂ on HIF-1 α protein levels, in the absence of any demonstrable effect on HIF-1 α mRNA levels, point to a mechanism involving decreased degradation of protein, rather than increased transcription. Most proteins are targeted for degradation by covalent modification with ubiquitin (25). The most notable example of cancer due to a defect in protein ubiquitination and degradation has been reported in von Hippel Lindau disease. The von Hippel Lindau gene (VHL) is a tumor suppressor gene. The VHL protein normally forms complexes with other proteins (elongin B, elongin C and Cul 2), which themselves form multi-protein complexes. These complexes target other proteins in the cell for ubiquitination. Two of the known proteins targeted for ubiquitination in the complex formed with the VHL protein are VEGF and HIF. When VHL is mutated, there is defective ubiquitination and degradation of both VEGF and HIF, with resultant tumors that are notably vascular (26,27).

Based upon our data from this first year of funding, we now hypothesize that PGE₂ is involved in the ubiquitination of the HIF-1 α protein. In the presence of hypoxia, HIF-1 α gene transcription and protein levels rise, and the protein binds to the promoter region of VEGF, thereby enhancing transcription of the VEGF gene. Hypoxia also increases COX-2 transcription and PGE₂ levels via an independent mechanism (activation of the NF- κ B pathway). We hypothesize that PGE₂ further increases HIF protein levels by decreasing its ubiquitination and degradation, thereby enhancing and prolonging the effects of hypoxia on VEGF gene expression. Over the next year, we will test this hypothesis by determining the effects of PGE₂ on HIF protein stability and ubiquitination.

These observations have important implications in prostate cancer therapy. Intratumoral hypoxia cannot be controlled. However, if these hypotheses are proven to be true, COX-2 inhibitors can help prevent hypoxic upregulation of tumor HIF and VEGF and can serve as effective antiangiogenic and anti-tumor therapy in human prostate cancer.

REFERENCES

1. Simmons D.L., Levy D.B., Yannoni Y., and Erikson, R.L. Identification of a phorbol ester-repressible *v-src*-inducible gene. *Proc. Natl. Acad. Sci. USA.* 86:1178-1182, 1989.
2. Kujubu, D.A., Flecher, B.S., Varnum B.C., Lim R.W., and Herschman H.R. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.* 166:12866-12872, 1991.
3. Tsujii M. and DuBois R.N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell.* 83:493-501, 1995.
4. Sheng H.M., Shao J.Y., Morrow J.D., Beauchamp R.D. and DuBois R.N. Modulation of apoptosis and bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res.* 58:362-366, 1998.
5. DuBois R.N., Giardello F.M. and Smalley W.E. Nonsteroidal anti-inflammatory drugs, eicosanoids and colorectal cancer prevention. *Gastroenterol. Clin. N. Am.* 25:773-791, 1996.
6. Sheng H., Shao J., Kirkland S.C., Isakson P., Coffey R., Morrow J., Beauchamp R.D. and DuBois R.N. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J. Clin. Invest.* 99:2254-2259, 1997.
7. Kawamori T., Rao C.V., Seibert K., and Reddy B.S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res.*, 58:409-412, 1998.
8. Auerbach, R. Angiogenesis-inducing factors: a review. In: Pick E., Ed., *Lymphokines*. New York, Academic Press, Vol 4:page 69, 1981
9. Folkman J. and Shing Y. Angiogenesis (Minireview). *J. Biol. Chem.* 267:10931-34, 1992.
10. Bicknell R., and Harris A.L. Novel growth regulatory factors and tumor angiogenesis. *Eur. J. Cancer* 27:781-785, 1991.
11. BenAv, P., Crofford L.J., Wilder R.L. and Hla T. Induction of VEGF expression in synovial fibroblasts by PGE and IL-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett.* 372:83-87, 1995.
12. Form D.M. and Auerbach R. PGE2 and angiogenesis. *Proc. Soc. Exp. Biol. Med.* 172:214-218, 1983.
13. Spisni E., Manica F., and Tomasi V. Involvement of prostanoids in the regulation of angiogenesis by polypeptide growth factors. *Prostaglandins, Leukotrienes, Essential Fatty Acids* 47:111-115, 1992.
14. Kirschenbaum A., Klausner A.P., Lee R., Unger P., Yao S., Liu X.H., and Levine A.C. Expression of cyclooxygenase-1 and cyclooxygenase-2 in the human prostate. *Urology* 56:671-676, 2000.
15. Liu X.-H., Yao S., Kirschenbaum A., and Levine A.C. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. *Cancer Res.* 58:4245-4249, 1998.
16. Liu X.-H., Kirschenbaum A., Yao S., Lee R., Holland J.F. and Levine A.C. Inhibition of cyclooxygenase-2 suppresses angiogenesis and the growth of prostate cancer cells in vivo. *J. Urology* 164:820-825, 2000 (**APPENDIX, Manuscript 1**).
17. Liu X.-H., Kirschenbaum A., Yao S., Stearns M.E., Holland J.F., Claffey K., and Levine A.C. Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line. *Clinical and Experimental Metastasis* 17:687-694, 1999 (**APPENDIX, Manuscript 2**).

18. Ferrara N., Houck K., Jakeman L., Leung D.W. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocrine Rev.* 13:18-32, 1992.
19. Plate K.H., Breier G., Weich H.A., Mennel H.D., Risau W. VEGF and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. *Int. J. Cancer* 59:520-529, 1994.
20. Soker S., Fidler H., Neufeld G., Klagsbrun M. Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF 165 via its exon 7-encoded domain. *J. Biol. Chem.* 271:5761-5767, 1996.
21. Kirschenbaum A., Wang J.-P., Ren M., Schiff J.D., Aaronson S.A., Droller M.J., Ferrara N., Holland J.F. and Levine A.C. Inhibition of vascular endothelial cell growth factor suppresses the in vivo growth of human prostate tumors. *Urol. Oncol.* 3:3-10, 1997.
22. Forsythe J.A., Jiang B.H., Iyer N.V., Agani F., Leung S.W., Koos R.D., Semenza G.L. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor I. *Molecular and Cellular Biology* 16:4604-4613, 1996.
23. Wenger R.H., Gassmann M. Oxygen(es) and the hypoxia-inducible factor I. *J. Biol. Chem.* 378:609-616, 1997.
24. Tjandrawinata R.R., Dahiya R., and Hughes-Fulford, M. Induction of cyclooxygenase-2 mRNA by prostaglandin E2 in human prostatic carcinoma cells. *Br. J. Cancer* 75:1111-1118, 1997.
25. Goldberg A.L., Elledge S.J., Harper W.J. The cellular chamber of doom. *Scientific American*, January issue:68-73, 2001.
26. Kaelin W.G. Many vessels, faulty gene. *Nature* 399:203-204.
27. Ohh M., Park C.W., Ivan M., Hoffman M.A., Kim T.Y., Huang L.E., Pavletich N., Chau V., Kaelin W.G. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat. Cell. Biol.* 2(7):423-427, 2000.

INHIBITION OF CYCLOOXYGENASE-2 SUPPRESSES ANGIOGENESIS AND THE GROWTH OF PROSTATE CANCER IN VIVO

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ABSTRACT

Purpose: Cyclooxygenase (COX)-2, an inducible enzyme which catalyzes the formation of prostaglandins from arachidonic acid, is expressed in prostate cancer specimens and cell lines. To evaluate the in vivo efficacy of a COX-2 inhibitor in prostate cancer, NS398 was administered to mice inoculated with the PC-3 human prostate cancer cell line.

Materials and Methods: A total of 28 male nude mice were inoculated subcutaneously with 1 million PC-3 cells. Tumors were palpable in all 28 animals 1 week after inoculation and mice were randomized to receive either vehicle (control) or NS398, 3 mg/kg. body weight, intraperitoneally three times weekly for 9 weeks. Tumors were measured at weekly intervals. After a 10-week experimental period, mice were euthanized and tumors were immuno-histochemically assayed for proliferation (PCNA), apoptosis (TUNEL) and microvessel density (MVD) (Factor-VIII-related antigen). Tumor VEGF content was assayed by Western blotting.

Results: NS398 induced a sustained inhibition of PC-3 tumor cell growth and a regression of existing tumors. Average tumor surface area from control mice was 285 mm.² as compared with 22 mm.² from treated mice (93% inhibition, $p < 0.001$). Immunohistochemical analysis revealed that NS398 had no effect on proliferation (PCNA), but induced apoptosis (TUNEL) and decreased MVD (angiogenesis). VEGF expression was also significantly down regulated in the NS398-treated tumors.

Conclusions: These results demonstrate that a selective COX-2 inhibitor suppresses PC-3 cell tumor growth in vivo. Tumor growth suppression is achieved by a combination of direct induction of tumor cell apoptosis and down regulation of tumor VEGF with decreased angiogenesis

KEY WORDS: Cyclooxygenase-2; Prostate cancer; Angiogenesis; Apoptosis; NS398

Prostate cancer is the most common malignancy and second leading cause of cancer-related deaths among men in the United States.¹ Although most patients with advanced prostate cancer respond to hormonal therapies, the majority relapse and eventually die of their disease. Conventional chemotherapeutic agents exhibit little activity against these advanced tumors. Thus, alternative approaches for the treatment of advanced prostate cancer are needed. It is well established that all tumors require the growth of new blood vessels to grow beyond 1 to 2 mm. and to metastasize.² We previously demonstrated that inhibition of tumor angiogenesis slows the growth of human prostate tumors in vivo.³ Therapies which have combined anti-tumor and anti-angiogenic effects should be effective in advanced disease.

Cyclooxygenase (COX), also referred to as prostaglandin endoperoxide synthase, is a key enzyme in the conversion of arachidonic acid to prostaglandins and other eicosanoids. Two isoforms of COX have been identified. COX-1 is expressed constitutively in many tissues and cell types, whereas COX-2 is inducible by a variety of factors, including cytokines, growth factors and tumor promoters.⁴ COX-2 is highly expressed in a number of human cancers and cancer cell lines, including prostate cancer.⁵ COX-2 was initially identified as one of early growth response genes^{6,7} which

affect a number of signal transduction pathways that modulate cellular adhesion, proliferation, apoptosis, and differentiation.^{8,9} More recently, several pieces of in vitro evidence have implied a linkage between COX-2 activity and cancer angiogenesis.^{10,11} These reports suggested that inhibition of COX-2 activity can serve as both anti-tumor and anti-angiogenic therapy.

Although inhibition of COX-2 activity by selective inhibitors appears to be effective in colon cancer prevention and treatment,^{12,13} very little attention has previously been given to COX-2 and its relationship to prostate cancer. We recently reported on the induction of apoptosis by a selective COX-2 inhibitor in the androgen-sensitive prostate cancer cell line, LNCaP in vitro.¹⁴ The present studies were undertaken to determine if NS398, a selective COX-2 inhibitor, is an effective anti-cancer agent in an in vivo model of human prostate cancer. We hypothesized that COX-2 inhibition would be even more efficacious in vivo than in vitro because of the added anti-angiogenic effects. Our data demonstrate that a selective COX-2 inhibitor, NS398, induces apoptosis in the androgen-independent PC-3 human prostate cancer cell line in vitro. Moreover, NS398 is a potent suppressor of PC-3 cell tumor growth in vivo with no indication of toxicity in nude mice. These effects were accomplished by a combination of induction of cancer cell apoptosis and suppression of tumor angiogenesis.

MATERIALS AND METHODS

Cell culture and viability assay. The PC-3 cell line was purchased from American Type Culture Collection (Rock-

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ville, MD). This cell line was routinely maintained in DMEM containing 10% FBS (Gibco-BRL, Grand Island, NY). For cell viability assays, PC-3 cells were plated at 1×10^6 cells/well in six-well cluster dishes with 2 ml. of 10% FBS-supplemented medium for 24 hours. At day 0, cells were washed with PBS, and the medium was changed to serum-free medium containing 0.1% BSA overnight. Incubations were continued with or without increasing concentrations of NS398 (Cayman Chemicals, Ann Arbor, MI) for 5 days with refeeding after 3 days. For the time course experiments, cells were incubated with or without 100 μ M NS398 and harvested at various time points. After 2 washes with PBS, cells were stained with trypan blue, and the number of living cells after treatment was determined by counting in a hemacytometer.

DNA fragmentation assay. DNA gel fragmentation assay was performed as previously described.¹⁴ Briefly, subconfluent cells were cultured in DMEM containing 10% FBS with or without NS398 (100 μ M) for various times. Cells were washed with cold PBS and lysed in lysis buffer (10 mM Tris, pH 7.4, 5 mM EDTA, 1% Triton X-100) for 20 minutes on ice. Micro-centrifugation was performed at 11,000 g for 20 minutes to separate the nuclear DNA precipitate from the fragmented DNA present in the supernatant. The supernatant was treated with 50 μ g./ml. RNase A at 37°C for 1 hour, and then proteinase K was added at 0.1 mg./ml. for another hour. After phenol-chloroform extraction, DNA from the supernatant was precipitated by ethanol and resuspended in 100 μ L of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA). Equal amounts of DNA samples (20 μ g.) were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining.

TUNEL assay. PC-3 cells were incubated in DMEM containing 10% FBS with or without NS398 for 3 days. Cells were washed with ice-cold PBS and harvested by centrifugation. For the assays on tumor tissues, tumor specimens from control versus NS398-treated mice were deparaffinized and washed in two changes of xylene for 5 minutes each wash, followed by washing in ethanol and PBS. Cells and tumor specimens were examined for apoptosis using the TUNEL method with the ApopTag in situ apoptotic detection kit (Oncor, Gaithersburg, MD) according to the manufacturer's instruction. The labeled cells and fixed tissues were examined using a fluorescent microscope.

Protein extraction from solid tumor and immunoblotting. Protein from solid PC-3 cell tumors were extracted using Tri-Reagent (Sigma, St. Louis, MO), according to the manufacturer's instruction. Protein content was measured with a protein assay kit (Bio-Rad, Hercules, CA). Samples were then electrophoresed on a 12% SDS-polyacrylamide gel, electrophoretically transferred to a polyvinylidene difluoride membrane (Dupont, Wilmington, DE), and incubated with anti-VEGF polyclonal antibodies (Santa Cruz BioTech, Santa Cruz, CA) overnight at 4°C. Secondary horseradish peroxidase-linked anti-rabbit IgG was used. Filters were developed by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). VEGF protein isoforms were identified as described by Houck et al.¹⁵ Relative protein expression was then quantitated with a densitometer (Molecular Dynamics, Sunnyvale, CA).

Animals and tumor cell inoculation. Male mice aged 6 to 8 weeks were used in the study. For tumor cell inoculation, subconfluent PC-3 cells were harvested with 0.1% trypsin. Ten million cells were suspended in 1 ml. of 10% FBS-containing DMEM (Gibco BRL, Gaithersburg, MD) and mixed with 1 volume of ice-cold Matrigel (Collaborative Biomedical Products, Bedford, MA). The cell-Matrigel suspension was allowed to warm up at room temperature for 5 minutes with gentle mixing and then inoculated subcutaneously into the inguinal region of each mouse.

Tumor growth in nude mice and NS398 administration.

From the second week of tumor cell inoculation, tumor bearing mice were randomly divided into two groups with 14 mice each, and received intraperitoneal injections of either vehicle as control or NS398 at the dose of 3 mg./kg. body weight three times a week. The dose of NS398 we used in this study was based on previous reports from Futaki et al.¹⁶ and Masferrer et al.,¹⁷ as well as our in vitro results. Animals were weighed and the tumor surface areas were measured with a vernier caliper at weekly intervals. The formula $(L/2) \times (W/2) \times \pi$ (where L is maximum diameter of each tumor, and W is the length at right angles to L) was used to calculate the tumor surface areas.¹⁸

Immunohistochemistry. Immunohistochemical staining for the expression of Factor VIII-related antigen and PCNA were carried out as previously described.^{19,20} Briefly, tumor specimens were deparaffinized, treated with 3% hydrogen peroxide, followed by incubation with the appropriate blocking serum and then incubated with the antibodies against factor VIII-related antigen (Boehringer Mannheim, Indianapolis, IN) for the detection of tumor microvessel density, or PCNA (Transduction Laboratory, Lexington, KY) for the detection of tumor cell proliferation. Staining was carried out using the avidin-biotin complex method with reagents from Vector Labs (Burlingame CA). The intensity and extent of positivity of every stained specimen was estimated by the percentage of positive staining cells scored under a light microscope on three separate occasions in a coded manner.

Measurement of microvessel density. Estimations of MVD were carried out as previously described.¹⁹ Briefly, microvessels were highlighted by staining endothelial cells for factor VIII-related antigen using a standard immunoperoxidase technique. MVD was assayed by light microscopy. Areas of highest neovascularization were identified by scanning the tumor sections at low magnifications (x 40 and x 100). After areas of highest neovascularization were identified, individual microvessel counts were made on a x 200 field (x 20 objective and x 10 ocular, 0.739 mm.² per field). Any brown-staining endothelial cell or endothelial cell cluster, clearly separate from adjacent microvessels, tumor cells, and other connective-tissue elements, was considered a single, countable microvessel. Results were examined by two blinded observers on three separate occasions in a coded manner. Data were expressed as the highest number of microvessels identified within any single x 200 field.

Statistics. Data are expressed as mean \pm SEM. Data from in vitro and in vivo were statistically analyzed with Student's unpaired t test.

RESULTS

In Vitro Studies. NS398 Decreases PC-3 Cell Viability: Initial experiments examined the effects of NS398 on PC-3 cell viability in vitro. As shown in fig. 1, A, treatment with 100 μ M NS398, significantly decreased the viability of PC-3 cells after 3 days (50% of control). PC-3 cell survival was 23% and 11% of control after 4 and 5 days treatment with NS398, respectively. The dose-dependence of the NS398 effect was next characterized. As shown in fig. 1, B, a modest decline in viable tumor cells was discernible after treatment with 10 μ M NS398 for 5 days. Higher concentrations of the inhibitor further decreased cell survival. These results indicate that NS398 decreases PC-3 cell survival in a time and dose-dependent fashion.

NS398-Induced PC-3 Cell Death Caused by Apoptosis: One of the possible mechanisms underlying the observed effect of NS398 on tumor cell viability is the direct induction of PC-3 cell apoptosis. To test this hypothesis, DNA fragmentation assay was performed. As shown in fig. 2, A, NS398-treated PC-3 cells produced a smear of different sizes of DNA fragments and a distinct oligosomal ladder, a typical characteristic of cells undergoing apoptosis. In contrast, control PC-3

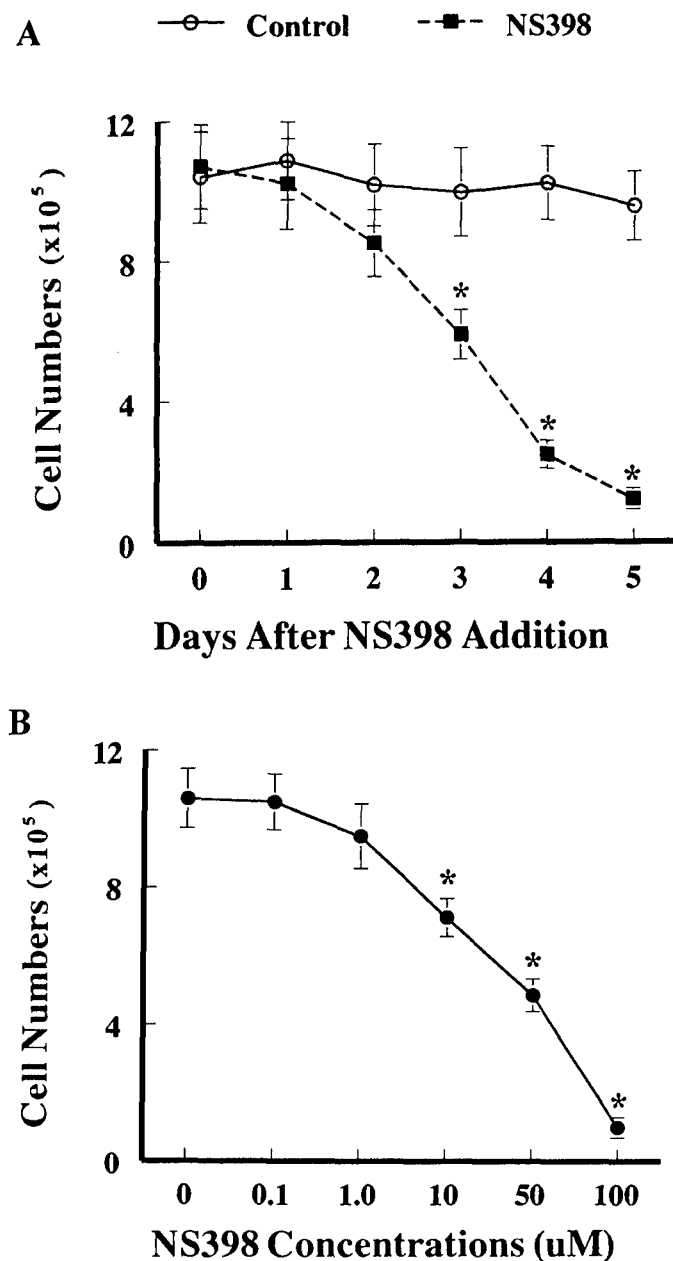


FIG. 1. NS398 decreases PC-3 cell viability. A, time course of cell viability over 5 days in the presence or absence of NS398. PC-3 cells were treated with 100 μ M NS398 for times indicated. B, dose-dependence of NS398 effects on cell viability. PC-3 cells were treated with increasing concentrations of NS398 for 5 days. After trypan blue staining, numbers of living cells were counted using hemacytometer. Data shown are means \pm SE of three independent experiments, * $p < 0.01$ versus control.

cells showed no evidence of detectable DNA fragments. The apoptosis induced by NS398 was further confirmed by TUNEL assay, in which the non-apoptotic cells are stained red while apoptotic cells stained yellow or green. As demonstrated in fig. 2, B, control PC-3 cells were red, whereas NS398-treated PC-3 cells stained yellow and green indicating apoptosis.

In Vivo Studies. NS398 Suppresses PC-3 Cell Tumor Growth in Nude Mice: The in vivo efficacy of NS398 was next determined in nude mice. As shown in fig. 3, A, over a 10-week experimental period, NS398, at a dose of 3 mg/kg, body weight given 3-times a week, produced a sustained inhibition of PC-3 cell tumor growth, and a regression of existing tumors beginning 2 weeks after treatment initiation.

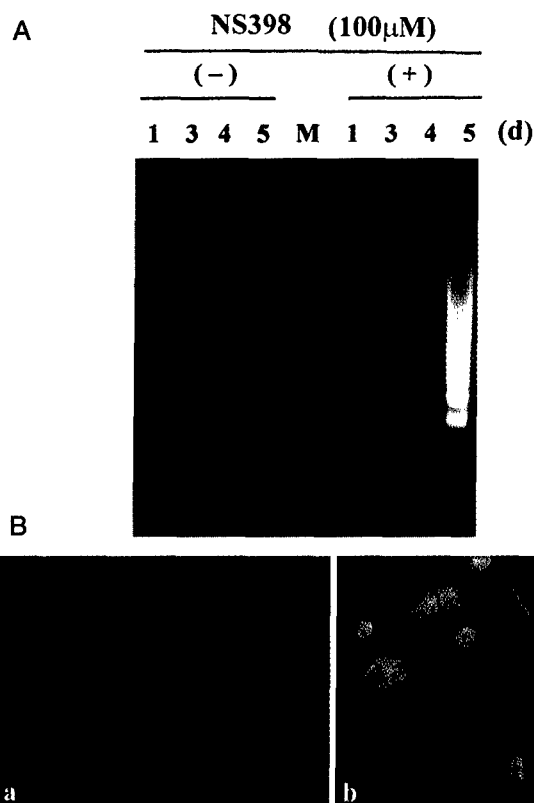


FIG. 2. NS398 induces apoptosis in PC-3 cells. Top, DNA fragmentation assay. PC-3 cells were treated with either vehicle as control or 100 μ M NS398 for days indicated. Cellular DNA was extracted and analyzed by agarose gel (1.2%) electrophoresis. Lane M is 100 bp DNA marker. Bottom, TUNEL assay. Cultured PC-3 cells treated with either vehicle as control (a) or 100 μ M NS398 (b) for 3 days. Apoptotic cells were detected by ApopTag in situ apoptosis detection kit, and examined by fluorescent microscope. Data shown are representative of three independent experiments.

Average tumor surface area at 9 weeks for the NS398 treatment was 22 mm.² versus 285 mm.² from control mice (>93% inhibition, $p < 0.001$). Tumors from three of fourteen NS398-treated mice were not palpable, indicating a complete regression of these tumors after drug administration. There was no evidence of drug-related toxicity in any of the mice. Three representative tumors which were randomly picked from control or NS398-treated mice are shown in fig. 3, B. Tumors from control mice are larger and more highly vascularized than those derived from drug-treated mice.

NS398 Induces PC-3 Tumor Cell Apoptosis and Inhibits Tumor Angiogenesis: We next determined the effect of NS398 on tumor cell proliferation, apoptosis and angiogenesis in vivo. As shown in fig. 4, immunohistochemical analysis of histological sections revealed that NS398 had no significant effect on tumor cell proliferation as detected by the staining with antibody against PCNA. NS398 treatment did, however, have a substantial effect on the induction of apoptosis in tumors derived from NS398-treated mice versus control detected by TUNEL assay.

Staining with Factor VIII-related antigen demonstrated a significant decrease (versus control) in microvessel density (MVD). Blood vessels in the tumors derived from control mice showed a sinusoidal pattern and well developed vascular networks. In contrast, the blood vessels in the tumors from NS398-treated mice consisted of randomly distributed endothelial cells which did not form organized vascular networks. Quantitative analysis of histological sections, as shown in fig. 5, revealed no change in the proliferative index (PCNA), but a 3.5-fold increase in the apoptotic index (TUNEL) and 3-fold decrease in MVD in the tumors derived from drug-treated versus control animals.

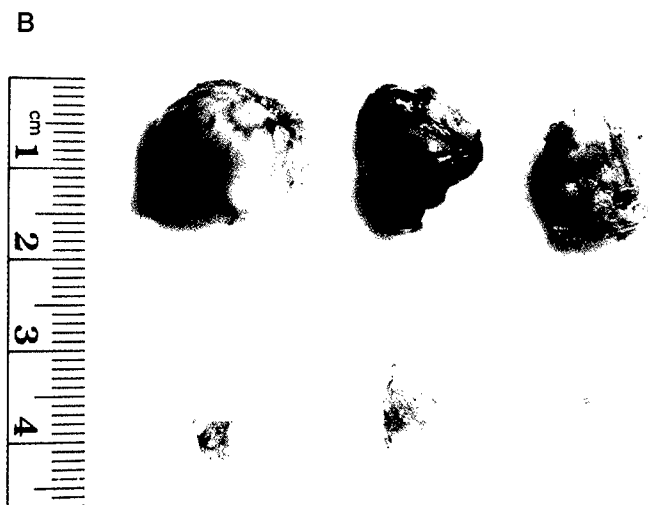
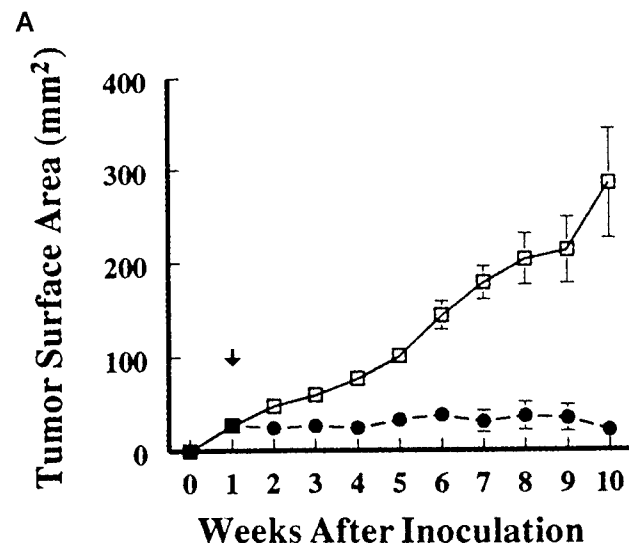


FIG. 3. NS398 suppresses PC-3 cell tumor growth in nude mice. *Top*, from second week of PC-3 cell inoculation, tumor bearing mice were randomized to receive i.p. injections of either vehicle as control (□) or NS398 (●), 3 mg/kg. body weight, three times weekly. Tumor surface areas were examined weekly. Vertical arrow indicates time for initiation of NS398 treatment. *Bottom*, three representative tumors collected from control mice (*top*) or NS398-treated mice (*bottom*) at 9 weeks after NS398 treatment.

NS398 Inhibits VEGF Production in PC-3 Cell Tumors: VEGF is a major stimulator of tumor angiogenesis.²¹ Our preliminary *in vitro* data have demonstrated that NS398 inhibited upregulation of VEGF by CoCl_2 -induced hypoxia (data not shown). To determine whether NS398 effects on tumor angiogenesis were due to suppression of VEGF, tumor tissues from control versus NS398-treated mice were assayed for the expression of VEGF protein isoforms. While VEGF₁₂₁ was barely detectable, VEGF₁₆₅ was the major band with a molecular weight of 21 kDa detected by Western blotting in PC-3 cell tumors (fig. 6, A). Quantitative analysis of VEGF₁₆₅ protein expression revealed a significant reduction (2.5-fold less than control, $p < 0.01$) in residual small tumors from NS398-treated mice after a 9-consecutive week treatment (fig. 6, B). VEGF₁₈₉ levels were also reduced in treated versus control animals (data not shown).

DISCUSSION

Overexpression of COX-2 may result in persistent activation of genes normally induced only transiently during passage through the cell cycle,^{9,22} and lead to phenotypic

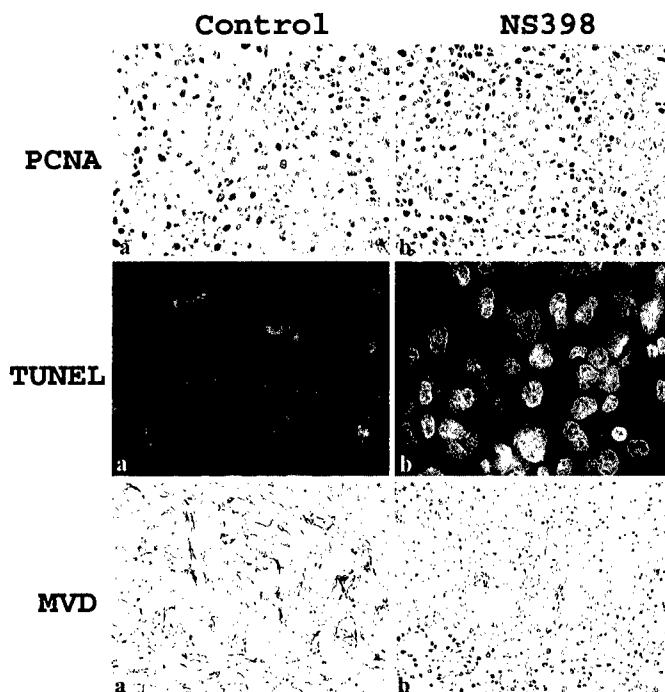


FIG. 4. Immunohistochemical analysis of histological sections of tumors derived from control versus NS398-treated mice. From second week of PC-3 cell inoculation, tumor bearing mice were randomized to receive i.p. injections of either vehicle as control or NS398 at dose of 3 mg/kg. body weight three times weekly for 9 weeks. Mice were then killed by CO_2 euthanasia, tumors were removed and processed to immunohistochemical analysis: PCNA for proliferative index, TUNEL for apoptotic index, and Factor VIII-related antigen for microvessel density.

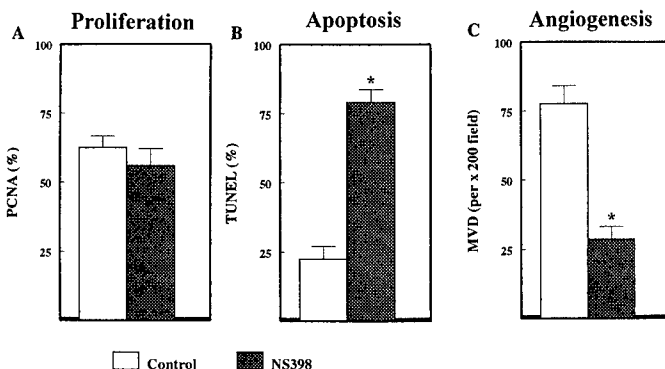


FIG. 5. NS398 induces tumor cell apoptosis and suppresses tumor angiogenesis. Histological sections of tumors from control ($n = 10$) versus NS398-treated ($n = 10$) mice were analyzed for A, proliferation (PCNA); B, apoptosis (TUNEL); and C, angiogenesis (MVD). Proliferative index and apoptotic index were estimated by percentage of cell scored under either light microscope or fluorescent microscope, at 200-fold magnification. MVD was determined by counting number of capillary blood vessels per x 200 field in sections stained with antibodies against Factor VIII-related antigen. * $p < 0.01$ versus control.

changes in these cells. COX-2 has been demonstrated to play a role in carcinogenesis,¹³ resistance of tumor cells to apoptosis, abnormal cell cycle regulation, cellular adhesion⁹ and tumor cell invasive capacity.²³ We have previously demonstrated that a selective COX-2 inhibitor induced apoptosis in an androgen-sensitive human prostate cancer cell line via inactivation of bcl-2.¹⁴ In the present study, we demonstrate that COX-2 inhibition also induces apoptosis, *in vivo*, and inhibits PC-3 cell tumor angiogenesis.

In the normal prostate, there is a balance between cell proliferation and cell death. It is well established that andro-

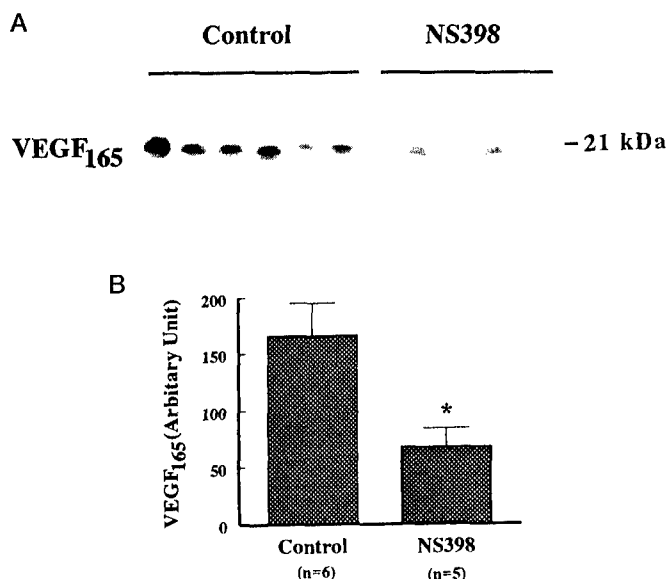


FIG. 6. NS398 inhibits VEGF production in PC-3 cell tumors. From second week of PC-3 cell inoculation, tumor bearing mice were randomized to receive i.p. injections of either vehicle as control or NS398 at the dose of 3 mg/kg. body weight three times weekly for 9 weeks. Mice were then killed by CO₂ euthanasia, tumors were removed. *Top*, protein was extracted and processed by Western blot analysis. Equal amounts (30 μ g.) of total protein were loaded in each lane. Blots were quantified by densitometry. *Bottom*, quantitative analysis of VEGF₁₆₅ protein expression in tumors derived from control versus NS398-treated mice. * $p < 0.01$ versus control.

gens have two effects on normal prostatic epithelial cells: stimulation of proliferation and prevention of apoptosis.²⁴ In prostate cancer, even in advanced, hormone refractory disease, the proliferative rate remains relatively low, but there is less apoptosis.²⁵ Therefore, our findings that a selective COX-2 inhibitor specifically induces apoptosis in both the androgen-sensitive LNCaP cell line¹⁴ and the androgen-insensitive PC-3 prostate cancer cell line (present report), offers a new therapeutic option which may be efficacious in biologically diverse prostate cancers.

Utilizing a selective COX-2 inhibitor, we herein demonstrate a reduction in prostate tumor microvessel density, a reflection of tumor angiogenesis. Angiogenesis is a central determinant of solid tumor growth.^{2,21} In prostate cancer, angiogenesis has been correlated with disease stage.¹⁹ We have previously demonstrated that inhibition of angiogenesis via the use of neutralizing antibodies to VEGF markedly reduces the growth of LNCaP prostate tumors in nude mice.³

In colon cancer, there is in vitro evidence that COX-2 is involved in the regulation of tumor angiogenesis.¹⁰ In the present report, we demonstrate that inhibition of COX-2 activity suppresses PC-3 prostate cancer angiogenesis in vivo, and that this effect is accompanied by decreases in tumor VEGF levels. Our in vitro studies with the PC-3ML cell line, a subline of PC-3, support the hypothesis that COX-2 plays a crucial role in hypoxia-induced upregulation of VEGF (unpublished data). Taken together, these results indicate that COX-2 inhibition can decrease prostate tumor angiogenesis, at least in part, via down regulation of VEGF production. Unfortunately, due to the small residual tumor size in NS398-treated mice, we were unable to examine the in vivo levels of other major angiogenic stimulators (bFGF, PDGF, TGF- β , endothelin-1, and inducible nitric oxide synthase), which have been reported to be upregulated by COX-2.¹⁰ It is also possible that COX-2 inhibitors upregulate the levels of some known angiogenesis inhibitors, such as thrombospondin²⁶ and angiostatin.²⁷

These data demonstrate that the systemic administration of a selective COX-2 inhibitor results not only in the sus-

tained inhibition of PC-3 tumor cell growth, but also dramatically reduces the size of existing tumors. These phenomenon can be explained by the combination of direct (induction of tumor cell apoptosis) and indirect (reduction of tumor VEGF with resultant decreased angiogenesis) effects of the inhibitor. These data provide a rationale for the use of currently available second generation COX-2 inhibitors²⁸ as safe and effective chemotherapeutic agents for the treatment of prostate cancer.

REFERENCES

- Parker, S. L., Tong, T., Bolden, S. and Wingo, P. A.: Cancer statistics, 1996. *CA Cancer J Clin*, **46**: 5, 1996
- Folkman, J.: What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst*, **82**: 4, 1992
- Kirschenbaum, A., Wang, J. P., Ren, M. Y., Schiff, S. A., Droller, M. J., Ferrara, N., Holland, J. F. and Levine, A. C.: Inhibition of vascular endothelial cell growth factor suppresses the in vivo growth of human prostate tumors. *Urol Oncology*, **3**: 3, 1997
- Herschman, H. R.: Primary response genes induced by growth factors and tumor promoters. *Ann Rev Biochem*, **60**: 281, 1991
- Takeuchi, M. M.: Cyclooxygenase-2 inhibitors in tumorigenesis (Part II). *J Natl Cancer Inst*, **90**: 1609, 1998
- Simmons, D. L., Levy, D. B., Yannoni, Y. and Erikson, R. L.: Identification of a phorbol ester-repressible *v-src*-inducible gene. *Proc Natl Acad Sci USA*, **86**: 1178, 1989
- Kujubu, D. A., Flecher, B. S., Varnum, B. C., Lim, R. W. and Herschman, H. R.: TIS10, a phorbol ester tumor promoter-inducible mRNA from swiss 3T3 cells encodes prostaglandin synthase/cyclooxygenase homologue. *J Biol Chem*, **266**: 12866, 1991
- Xie, W. L., Robertson, D. L. and Simmons, D. L.: Mitogen-inducible prostaglandin G/H synthase: a target for NSAIDs. *Drug Dev Res*, **25**: 249, 1991
- Tsuji, M., DuBois, R. N.: Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell*, **83**: 493, 1995
- Tsuji, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M. and DuBois, R. N.: Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell*, **93**: 705, 1998
- Schmedt, J. F., Jr., Ji, Y. S., Liu, W. L., DuBois, R. N. and Runge, M. S.: Hypoxia induces cyclooxygenase-2 via the NF- κ B p65 transcription factor in human vascular endothelial cells. *J. Biol. Chem.*, **272**: 601, 1997
- Sheng, X., Shao, J., Kirkland, S. C., Isakson, P., Coffey, R., Morrow, J., Beauchamp, R. D. and DuBois, R. N.: Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J Clin Invest*, **99**: 2254, 1997
- Kawamori, T., Rao, C. V., Seibert, K. and Reddy, B. S.: Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res*, **58**: 409, 1998
- Liu, X. H., Yao, S., Kirschenbaum, A. and Levine, A. C.: NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulation bcl-2 expression in LNCaP cells. *Cancer Res*, **58**: 4245, 1998
- Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B. and Leung, D. W.: The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol*, **5**: 1806, 1991
- Putaki, N., Yoshikawa, K., Hamasaka, Y., Arai, I., Higuchi, S., Iizuka, H. and Otomo, S.: NS398, a novel non-steroidal anti-inflammatory drug with potent analgesic and antipyretic effects, which causes minimal stomach lesions. *Gen Pharmacol*, **24**: 105, 1993
- Masferrer, J. L., Zweifel, B. S., Manning, P. T., Hauser, S. D., Leahy, K. M., Smith, W. G., Isakson, P. C. and Seibert, K.: Selective inhibition of inducible cyclooxygenase-2 in vivo is antiinflammatory and nonulcerogenic. *Proc Natl Acad Sci USA*, **91**: 3228, 1994
- Rose, D. P., Connolly, J. M. and Meschter, C. L.: Effect of dietary fat on human breast cancer growth and metastasis in nude mice. *J Natl Cancer Inst*, **83**: 1491, 1991
- Weidner, N., Carroll, P. R., Flax, J., Blumenfeld, W. and Folkman, J.: Tumor angiogenesis correlates with metastasis in

- invasive prostate carcinoma. *Am J Pathol*, **143**: 401, 1993
20. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R. and Folkman, J.: Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*, **88**: 277, 1997
 21. Ferrara, N. and Davis-Smyth, T.: The biology of vascular endothelial growth factor. *Endocrine Rev*, **18**: 4, 1997
 22. Han, J. W., Sadowski, H., Young, D. A. and Macara, I. G.: Persistent induction of cyclo-oxygenase in p60^{src}-transformed 3T3 fibroblasts. *Proc Natl Acad Sci USA*, **87**: 3373, 1990
 23. Tsujii, M., Kawano, S. and DuBois, R. N.: Cyclooxygenase-2 expression in colon cancer cells increases metastatic potential. *Proc Natl Acad Sci USA*, **94**: 3336, 1997
 24. Denmeade, S. R., Lin, X. S. and Isaacs, J. T.: Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. *Prostate*, **28**: 2516, 1996
 25. Berges, R. S., Vukanovic, J., Epstein, J. I., Carmichel, M., Cisek, L., Johnson, D. E., Veltri, R. W., Walsh, P. H. and Isaacs, J. T.: Implication of cell kinetic changes during the progression of human prostatic cancer. *Clin Cancer Res*, **1**: 473, 1995
 26. Dameron, K. M., Volpert, O. V., Tainsky, M. A. and Bouck, N.: Control of angiogenesis in fibroblast by p53 regulation of thrombospondin-1. *Science*, **265**: 1582, 1994
 27. O'Reilly, M. S.: Angiostatin: an endogenous inhibitor of angiogenesis and of tumor growth. *EXS*, **79**: 273, 1997
 28. Kalgutkar, A. S., Crews, B. C., Rowlinson, S. W., Garner, C., Seibert, K., Marnett, J.: Aspirin-like molecules that covalently inactivate cyclooxygenase-2. *Science*, **280**: 1268, 1998



Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line

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Key words: angiogenesis, cyclooxygenase-2, cobalt chloride-simulated hypoxia, metastasis, NS398, prostaglandin E₂, prostate cancer, vascular endothelial growth factor

Abstract

Upregulation of vascular endothelial growth factor (VEGF) expression induced by hypoxia is crucial event leading to neovascularization. Cyclooxygenase-2, an inducible enzyme that catalyzes the formation of prostaglandins (PGs) from arachidonic acid, has been demonstrated to be induced by hypoxia and play role in angiogenesis and metastasis. To investigate the potential effect of COX-2 on hypoxia-induced VEGF expression in prostate cancer. We examined the relationship between COX-2 expression and VEGF induction in response to cobalt chloride (CoCl₂)-simulated hypoxia in three human prostate cancer cell lines with differing biological phenotypes. Northern blotting and ELISA revealed that all three tested cell lines constitutively expressed VEGF mRNA, and secreted VEGF protein to different degrees (LNCaP > PC-3 > PC3ML). However, these cell lines differed in the ability to produce VEGF in the presence of CoCl₂-simulated hypoxia. CoCl₂ treatment resulted in 40% and 75% increases in VEGF mRNA, and 50% and 95% in protein secretion by LNCaP and PC-3 cell lines, respectively. In contrast, PC-3ML cell line, a PC-3 subline with highly invasive, metastatic phenotype, exhibits a dramatic upregulation of VEGF, 5.6-fold in mRNA and 6.3-fold in protein secretion after treatment with CoCl₂. The upregulation of VEGF in PC-3ML cells is accompanied by a persistent induction of COX-2 mRNA (6.5-fold) and protein (5-fold). Whereas COX-2 expression is only transiently induced in PC-3 cells and not affected by CoCl₂ in LNCaP cells. Moreover, the increases in VEGF mRNA and protein secretion induced by CoCl₂ in PC-3ML cells were significantly suppressed following exposure to NS398, a selective COX-2 inhibitor. Finally, the effect of COX-2 inhibition on CoCl₂-induced VEGF production was reversed by the treatment with exogenous PGE₂. Our data demonstrate that VEGF induction by cobalt chloride-simulated hypoxia is maintained by a concomitant, persistent induction of COX-2 expression and sustained elevation of PGE₂ synthesis in a human metastatic prostate cancer cell line, and suggest that COX-2 activity, reflected by PGE₂ production, is involved in hypoxia-induced VEGF expression, and thus, modulates prostatic tumor angiogenesis.

Introduction

Angiogenesis is a critical element for solid tumor growth and metastasis [1]. One of the factors involved in neovascularization is vascular endothelial growth factor (VEGF). VEGF plays a key role in both normal vasculogenesis and angiogenesis in many disease states. Upregulation of VEGF expression is a major event leading to promotion of angiogenesis in malignancy. In solid tumors, VEGF is mainly regulated by hypoxia via hypoxia inducible factor-1 (HIF-1) [2, 3]. However, recent report indicated that HIF-1 does

not appear to be sufficient for the full induction of VEGF in response to hypoxia. Unknown cellular factor(s) other than HIF-1 is required in this process [4, 5].

Cyclooxygenase (COX)-2 is an inducible enzyme required in the conversion of prostaglandins (PGs) from arachidonic acid. It is highly expressed in a number of human cancers and cancer cell lines, including prostate cancers [6, 7]. Overexpression and persistent existence of COX-2 has been linked to promotion of tumorigenesis, resistant to apoptosis, abnormal cell cycle regulation [8], and a feature of the aggressive, metastatic phenotype of human cancer cells [9, 10]. More recently, several pieces of evidence have implied a linkage between COX-2 activity and hypoxia-induced cancer angiogenesis [11, 12]. In addition, prostaglandin E₂ (PGE₂), a major COX-2 derived product, is

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reported to be a stimulator of angiogenesis [13]. These data suggest a novel approach to cancer treatment by inhibition of COX-2 activity.

Prostate cancer is the most common cancer and second leading cause of cancer death in US males. A number of clinical investigations have demonstrated a relationship between the degree of neovascularization and the progression of prostatic cancer grade [14], metastatic behavior [15], and cancer-specific survival [16]. While several reports have suggested an association between high degrees of VEGF expression and tumor microvessel density, a measure of angiogenesis, in breast and lung cancers [17, 18], it is not the case in human prostate cancer cell lines. Rose et al. reported that VEGF expression, at least in the generally available human prostate cancer cell lines, does not correlate with their capacity for invasion and metastasis, or with the degree of angiogenesis evident *in vivo* [19, 20]. To determine the relationships between hypoxic regulation of VEGF expression and metastatic phenotype of prostate cancer cells, and investigate the potential effect of COX-2 on hypoxia-induced VEGF expression. The present study compared the expression of COX-2 and VEGF with or without cobalt chloride treatment in three human prostate cancer cell lines, i.e., LNCaP, an androgen-sensitive non-invasive cell line; PC-3, an androgen-independent, less invasive, and weakly metastatic cell line; and PC-3ML, a highly invasive, metastatic (bone-targeting) PC-3 subline. Cobalt chloride treatment of cells *in vitro* has previously been shown to induce cellular changes which are similar to those seen after hypoxia [2, 21]. In addition, the effects of a selective COX-2 inhibitor and exogenous PGE₂ on the CoCl₂-induced VEGF production were evaluated in these cell lines.

Materials and methods

Cell culture

LNCaP and PC-3 cell lines were purchased from American Type Culture Collection (ATCC, Rockville, Maryland). PC-3ML, a PC-3 subline with highly invasive potential *in vitro* and bone-targeting metastasis in SCID mice, was established by Dr. Mark E. Stearns (MPC-Hahnemann University, Philadelphia, Pennsylvania) [22]. LNCaP cells were maintained in RPMI 1640 medium, while PC-3 and PC-3ML cells were cultured in DMEM with 10% FBS. Hypoxia-like conditions were chemically created by exposure of cells to 100 μ M cobalt chloride (CoCl₂, Sigma, St. Louis, Missouri). Before treatment with various compounds, i.e., CoCl₂, NS398 (Cayman Chemical Co., Ann Arbor, Michigan) and PGE₂ (Oxford, BioMed, Inc., Oxford, Michigan), subconfluent cells were washed twice with PBS and changed to serum-free medium containing 0.1% BSA for 24 h.

Immunoblotting

Cells cultured under the desired conditions were lysed as described previously [9]. Briefly, cells were rinsed twice

with ice-cold PBS and scraped with 1.5 ml of PBS containing 4 mM iodoacetate. After centrifugation, the pellets were resuspended in 50 μ l of CHAPS extraction solution (10 mM CHAPS, 2 mM EDTA, pH 8.0, and 4 mM iodoacetate in PBS) with protease inhibitors. The samples were then incubated for 30 min on ice and centrifuged at 15,000 g for 10 min. The supernatants were electrophoresed on a 9% SDS-polyacrylamide gel, electrophoretically transferred to a polyvinylidene difluoride membrane (DuPont, NEN), and incubated with anti-COX-2 antibodies (Oxford BioMed, Inc.) for 2 h at room temperature. Secondary horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham, Arlington Heights, Illinois) was used. Filters were developed by the enhanced chemiluminescence system (Amersham).

Northern blotting

Total RNA extraction and Northern blotting were carried out as previously described [9]. Briefly, subconfluent cells cultured under desired conditions were harvested, and total RNA was isolated and fractionated on a 1.2% agarose-formaldehyde gel. After transfer to a nitrocellulose membrane, hybridization was performed with an [α -³²P]-labeled either COX-2 (Oxford BioMed, Inc.) or VEGF cDNA (encoding the 165 amino acid isoform of VEGF, a gift from Dr. Kevin Claffey, Beth Israel Deaconess Medical Center, Boston, Massachusetts) probe. The membranes were subsequently hybridized with a β -actin cDNA probe to monitor RNA loading.

Preparation of condition medium for ELISA

Condition medium was prepared as described previously [23]. Briefly, Cells were plated at 1×10^5 cells/well in six-well cluster dishes with 2 ml medium containing 10% FBS. After washing with PBS, serum-free medium was replaced. Incubations were continued under the desired conditions with refeeding after 3 days. Cells were cultured for another 24 h, medium was collected, centrifuged at 800 g for 10 min to remove suspended cells, and stored at -70°C for assays. ELISA kits for VEGF detection were purchased from R&D Systems (Minneapolis, Minnesota), and the kits for PGE₂ assay were obtained from Oxford BioMed, Inc. The results were normalized to cell number.

Results

CoCl₂-induced upregulation of VEGF in prostate cancer cell lines

Initial studies examined the expression of VEGF mRNA and protein secretion in LNCaP, PC-3, and PC-3ML cells in the presence or absence of CoCl₂. As shown in Figure 1A, although all three cell lines expressed constitutive levels of VEGF mRNA (LNCaP > PC-3 > PC3ML), and responded to CoCl₂, the degrees of the induction significantly varied. CoCl₂ induced 40% and 75% increase in VEGF mRNA in LNCaP and PC-3 cells, respectively. In contrast, PC-3ML

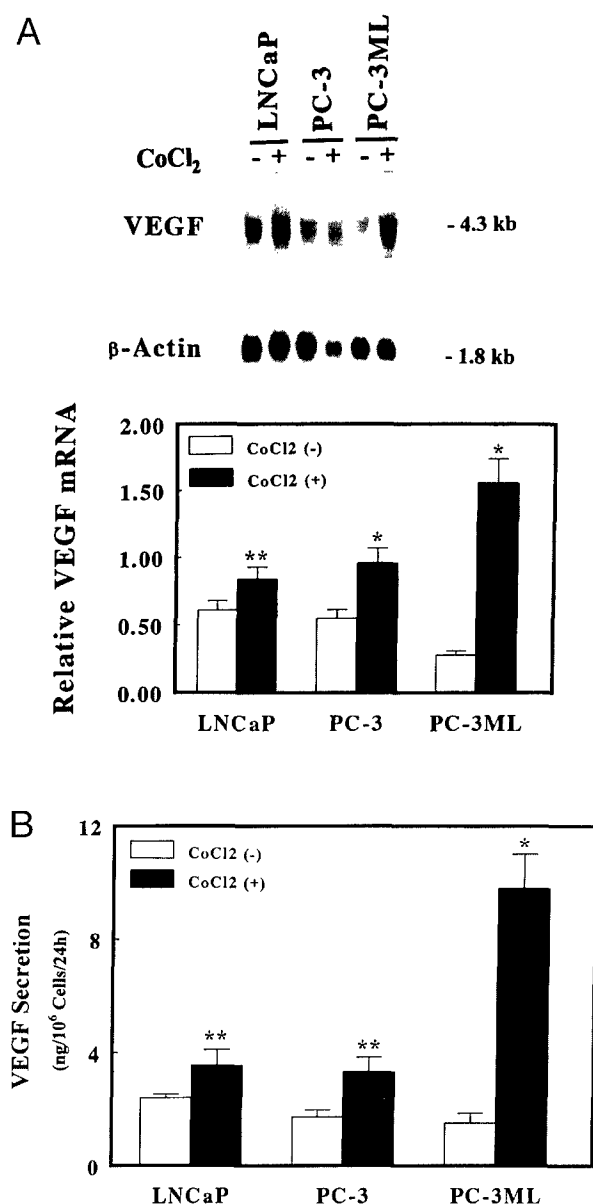


Figure 1. CoCl₂-induced upregulation of VEGF in prostate cancer cell lines. (A) CoCl₂-induced upregulation of VEGF mRNA. Prostate cancer cells were cultured in serum-free medium and treated with or without 100 μ M CoCl₂ for 2 d. Total RNA was then extracted and subjected to Northern blot analysis with a VEGF cDNA probe followed by rehybridization with a β -actin probe, and the amount of specific RNA was quantitated by densitometry. The result shown in upper panel is a representative from triplicate experiments. The quantitative analysis of VEGF mRNA expression from three independent experiments is shown in lower panel. Data are expressed as ratio of VEGF to β -actin mRNA, and are means \pm SEM. (B) CoCl₂-induced upregulation of VEGF protein secretion. Prostate cancer cells were incubated in serum-free medium and treated with or without 100 μ M CoCl₂ for 2 d. Culture medium was then collected, clarified, and subjected to ELISA to quantitate VEGF protein secretion. The results were normalized to cell number. The data represent mean \pm SEM from three separate determinations. ** P < 0.05, * P < 0.01 vs. control.

cell line was distinguished from the others by a dramatic up-regulation of VEGF mRNA (5.6-fold) in response to CoCl₂ despite of the low basal level. VEGF protein secretion was next examined with or without CoCl₂ treatment in these cell lines. As shown in Figure 1B, ELISA revealed that basal level of VEGF protein secretion is highest in LNCaP among the tested three cell lines, but only a 50% inducible level was detected following the addition of CoCl₂. In contrast, CoCl₂ induced 95% and 6.3-fold increase in VEGF protein secretion in PC-3 and PC-3ML cell lines, respectively.

CoCl₂-induced upregulation of COX-2 expression and PGE₂ production in prostate cancer cell lines

The expression of COX-2 mRNA, protein and PGE₂ production in LNCaP, PC-3, and PC-3ML cell lines and their responsiveness to CoCl₂ were next assayed. As shown in Figure 2A, LNCaP cells expressed a low level of COX-2 mRNA which was not influenced by CoCl₂. In contrast, PC-3, and PC-3ML cells expressed a relative high level of COX-2 mRNA. With the addition of CoCl₂, COX-2 mRNA was induced within 12 h in both PC-3 and PC-3ML cell lines. However, the induction of COX-2 in PC-3 cells was transient (with a peak of 1.6-fold induction), and the level returned to basal level after 24 h despite the continued presence of CoCl₂. Whereas COX-2 expression was persistently induced with a continuous enhancement after initiation in PC-3ML cells. There was a 6.5-fold increase by 72 h after CoCl₂ administration. Western blot analysis was next performed to determine whether the difference in the expression and CoCl₂-induced COX-2 mRNA in these cell lines influences their protein expression. Figure 2B demonstrates a low expression level of COX-2 protein which did not respond to hypoxic stimulation in LNCaP cells. In contrast, while CoCl₂ induced a 1.4-fold increase in COX-2 protein expression within 16 h which was declined to basal level by 48 h after exposure in PC-3 cells. COX-2 protein expression was upregulated by CoCl₂ within 16 h with an additional increase (maximum induction of 5-fold) that was sustained over a 72 h incubation period under CoCl₂-simulated hypoxia. COX-2 enzymatic activity, as reflected by PGE₂ synthesis was also determined. ELISA revealed a low level of PGE₂ secretion which stayed constant in the presence of CoCl₂ in LNCaP cells. The regulation of PGE₂ production by CoCl₂ in PC-3 and PC-3ML cell lines was consistent with that of COX-2 expression. Although both PC-3 and PC-3ML cell lines responded to CoCl₂ stimulation. The induction pattern of PGE₂ production was significantly different. CoCl₂ only induced a transient enhancement of PGE₂ secretion in PC-3 cells. Whereas PGE₂ secretion was persistently up-regulated with a 6-fold induction seen by 72 h after CoCl₂ addition in PC-3ML cells (Figure 2C).

Effect of COX-2 inhibition on CoCl₂-induced VEGF production

The co-induction of VEGF and COX-2 by CoCl₂ in PC-3 and PC-3ML cells let us further investigated the potential effect of COX-2 activity on the upregulation of VEGF in

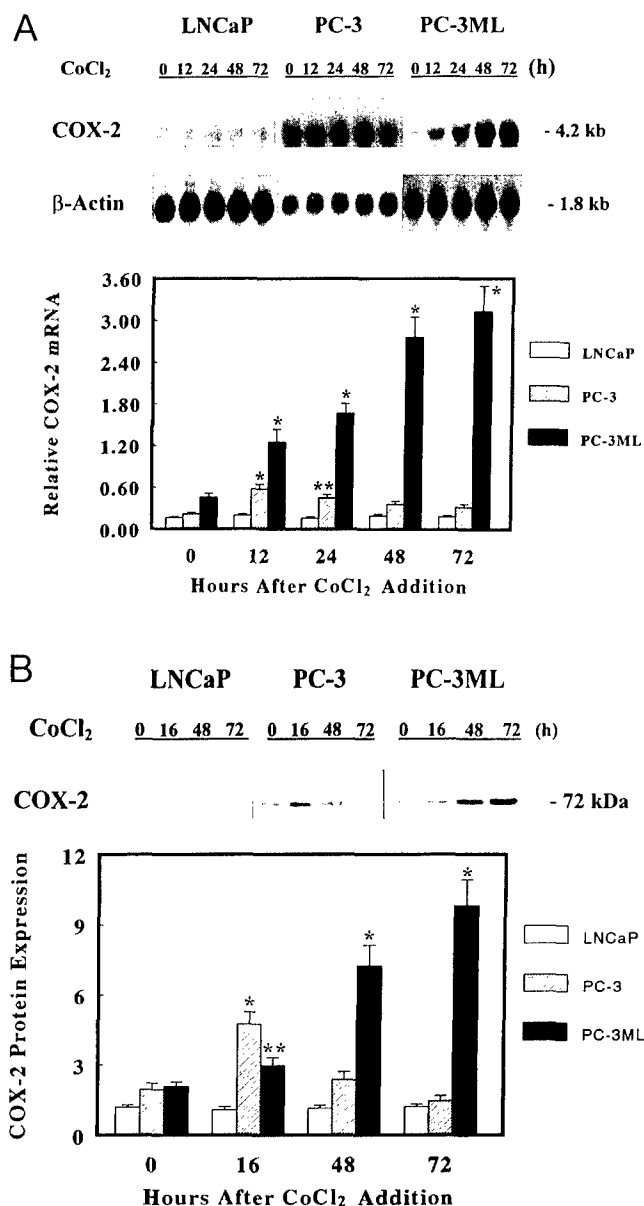


Figure 2. CoCl₂-induced upregulation of COX-2 expression and PGE₂ production in prostate cancer cell lines. (A) CoCl₂ upregulated COX-2 mRNA expression. Prostate cancer cells were cultured in serum-free medium and treated with or without 100 μ M CoCl₂ for the times as indicated. Total RNA was extracted at the end of each time points, and subjected to Northern blot analysis with a COX-2 cDNA probe followed by rehybridization with a β -actin probe the amount of specific RNA was quantitated by densitometry. The result shown in upper panel is a representative from triplicate experiments. The quantitative analysis of COX-2 mRNA expression from three independent experiments is shown in lower panel. The results are expressed as ratio of COX-2 to β -actin mRNA, and are means \pm SEM. ** P < 0.05, * P < 0.01 vs. control. (B) CoCl₂ upregulated COX-2 protein expression. Prostate cancer cells were incubated in serum-free medium and treated with or without 100 μ M CoCl₂ for the times as indicated. Protein was extracted at the end of each time points, and subjected to Western blot analysis with a polyclonal anti-COX-2 antibody. The amount of COX-2 protein expression was quantitated by densitometry. The result shown in upper panel is a representative from triplicate experiments. The quantitative analysis of COX-2 protein expression from three independent experiments is shown in lower panel. The results are expressed as arbitrary unit, and are means \pm SEM. ** P < 0.05, * P < 0.01 vs. control. (C) CoCl₂ induced upregulation of PGE₂ production. Prostate cancer cells were incubated in serum-free medium and treated with or without 100 μ M CoCl₂ for the times as indicated. Culture medium was collected, clarified, and subjected to ELISA to quantitate PGE₂ secretion. The results were normalized to cell number. The data represent mean \pm SEM from three separate determinations. ** P < 0.05, * P < 0.01 vs. control.

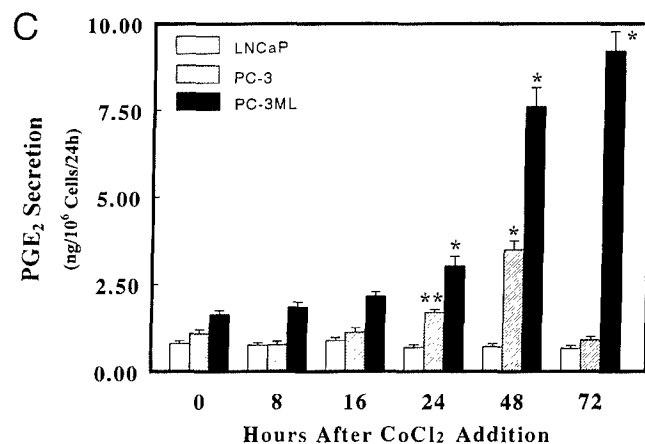


Figure 2. Continued.

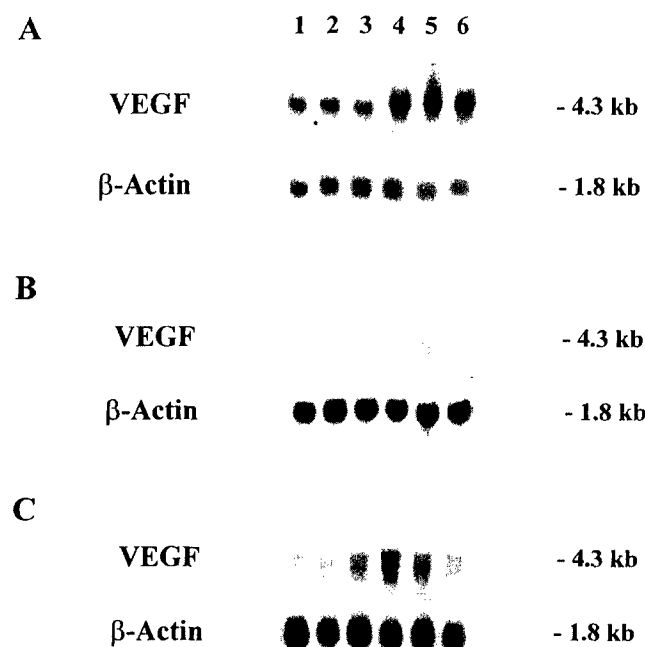


Figure 3. Effect of NS398 on the induction of VEGF mRNA by CoCl₂ in prostate cancer cell lines. Prostate cancer cells (A) LNCaP (B) PC-3 and (C) PC-3ML were cultured in serum-free medium in the absence (lanes 1–3) or presence (lanes 4–6) of 100 μ M CoCl₂ for 2 d. Cells were also treated with vehicle as control (lanes 1, 4), 10 μ M NS398 (lanes 2, 5), or 100 μ M NS398 (lanes 3, 6). Total RNA was then extracted and subjected to Northern blot analysis with a VEGF cDNA probe, followed by rehybridization with a β -actin probe. A representative result from triplicate experiments is shown.

response to CoCl₂ by employment of a selective COX-2 inhibitor, NS398. Northern blot analysis demonstrated that NS398 had no significant effect on VEGF mRNA expression in the absence of CoCl₂ in LNCaP and PC-3 cells, but a slight upregulation in PC-3ML cells. This enhanced expression of VEGF mRNA may, presumably, be due to the inhibition of the COX-2 metabolic pathway which converts arachidonic acid to prostaglandins. Arachidonic acid may then be metabolized by the lipoxygenase pathway to produce HETEs and leukotrienes leading to an activation of the lipoxygenase pathway. The lipoxygenase pathway has also been shown to be involved in prostate cancer angiogenesis and tumor growth [24]. However, in the presence

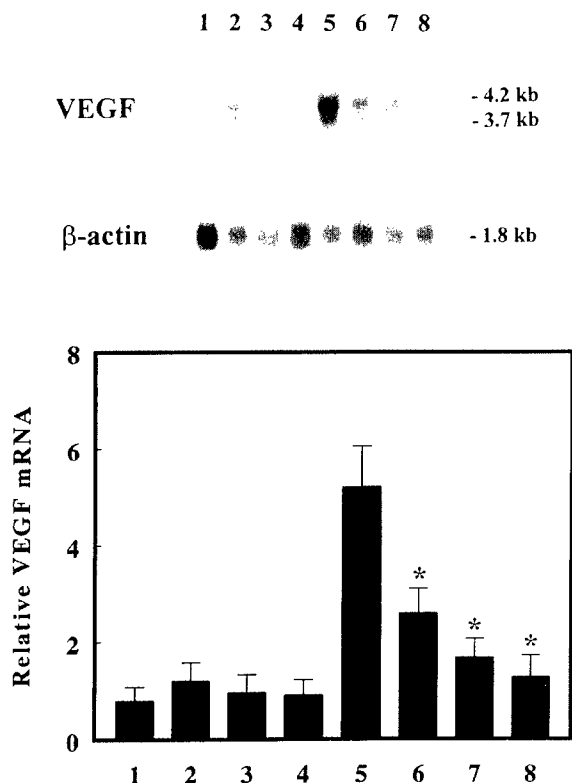


Figure 4. Time-dependent inhibition of VEGF mRNA by NS398 in CoCl₂-treated PC-3ML cells. PC-3ML cells were incubated in serum-free medium in the absence (lanes 1–4) or presence (lanes 5–8) of 100 μ M CoCl₂. Cells were also treated with vehicle as control (lanes 1, 5), 100 μ M NS398 for 1 day (lanes 2, 6), 2 days (lanes 3, 7) or 3 days (lanes 4, 8). Total RNA was then extracted and subjected to Northern blot analysis with a VEGF cDNA probe, followed by rehybridization with a β -actin probe. A representative result from duplicate experiments is shown. The quantitative analysis of VEGF mRNA expression from two independent experiments is shown in lower panel. Data are expressed as ratio of VEGF to β -actin mRNA, and are means \pm SEM. * $P < 0.01$ vs. the cells treated with CoCl₂ only.

of CoCl₂, NS398 did not influence the expression of VEGF mRNA by LNCaP cells after a 2-day treatment with 100 μ M CoCl₂ (Figure 3A). In contrast, VEGF mRNA expression induced by CoCl₂ was partially blocked by the addition of NS398 at the dose of 100 μ M for 2d in PC-3 cells (Figure 3B). The significant effect of NS398 on this process was observed only in PC-3ML cells. As shown in Figure 3C, the induced VEGF mRNA expression by CoCl₂ in PC-3ML cells was blocked by the addition of increasing concentrations of NS398. Figure 4 demonstrates that NS398-abolished upregulation of VEGF mRNA induced by CoCl₂ in PC-3ML cells was time dependent with maximum blockade of CoCl₂ effect seen 48 h following 100 μ M NS398 treatment. The effect of NS398 on the induction of VEGF protein secretion by CoCl₂ in PC-3ML cells was next determined using ELISA. The time course studies reveal that the suppression of CoCl₂ effects begins at 16 h after NS398 addition and was sustained over a 72 h incubation period in continuous presence of CoCl₂ (Figure 5A). The dose-dependence experiments demonstrated that NS398 produced a similar blockade of CoCl₂ effects on VEGF protein as that seen at the mRNA level (Figure 5B).

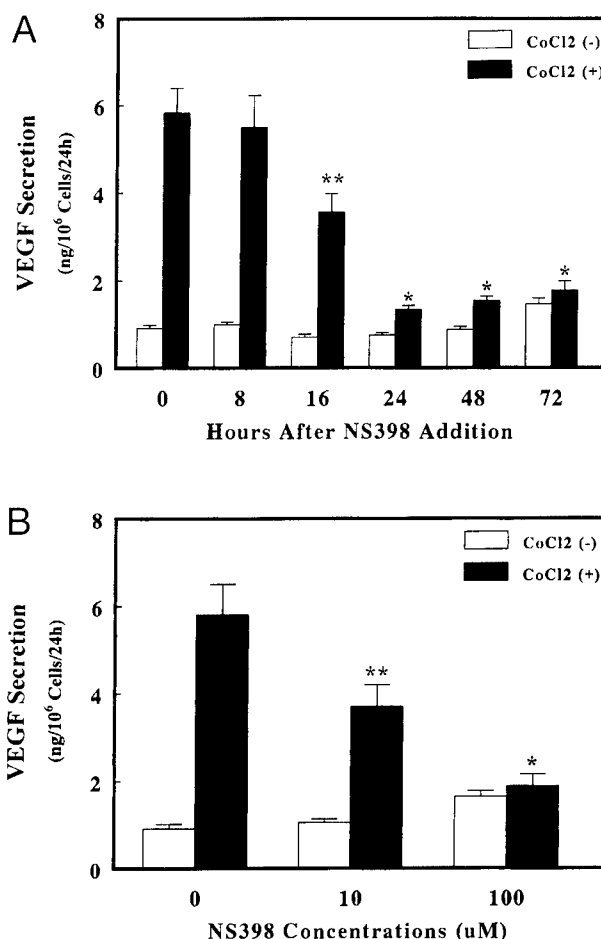


Figure 5. Inhibition of CoCl₂-induced VEGF protein secretion by NS398 in PC-3ML cells. (A) Time-dependent inhibition of VEGF protein secretion by NS398 in CoCl₂-treated PC-3ML cells. Cells were incubated in serum-free medium in the presence or absence of 100 μ M CoCl₂. Cells were also treated with vehicle as control (as indicated by zero) or 100 μ M NS398 for various times as indicated. (B) Dose-dependent inhibition of CoCl₂-induced VEGF protein secretion by NS398. Cells were incubated in serum-free medium in the presence or absence of 100 μ M CoCl₂. Cells were also treated with vehicle as control (as indicated by zero) or various concentrations of NS398 as indicated for 2 d. Culture medium was then collected, clarified, and subjected to ELISA to quantitate VEGF secretion. The results were normalized to cell number. The data represent mean \pm SEM from three separate determinations. ** $P < 0.05$, * $P < 0.01$ vs. control.

Effect of COX-2 inhibition on CoCl₂-induced VEGF production can be reversed by PGE₂ treatment

Because CoCl₂-induced VEGF expression was suppressed by treatment with NS398, we next sought to determine whether this inhibition could be overcome by addition of exogenous PGE₂, the major COX-2-derived eicosanoid product. As shown in Figure 6A, Northern blot analysis demonstrated that inhibition of CoCl₂-induced VEGF mRNA expression in the presence of NS398 was reversed by the addition of exogenous PGE₂ to the PC-3ML cells. Similar responses in VEGF protein secretion were observed in cultured PC-3ML cells (Figure 6B). These results indicate that PGE₂ produced via COX-2 pathway plays a crucial role in VEGF upregulation by CoCl₂-simulated hypoxia.

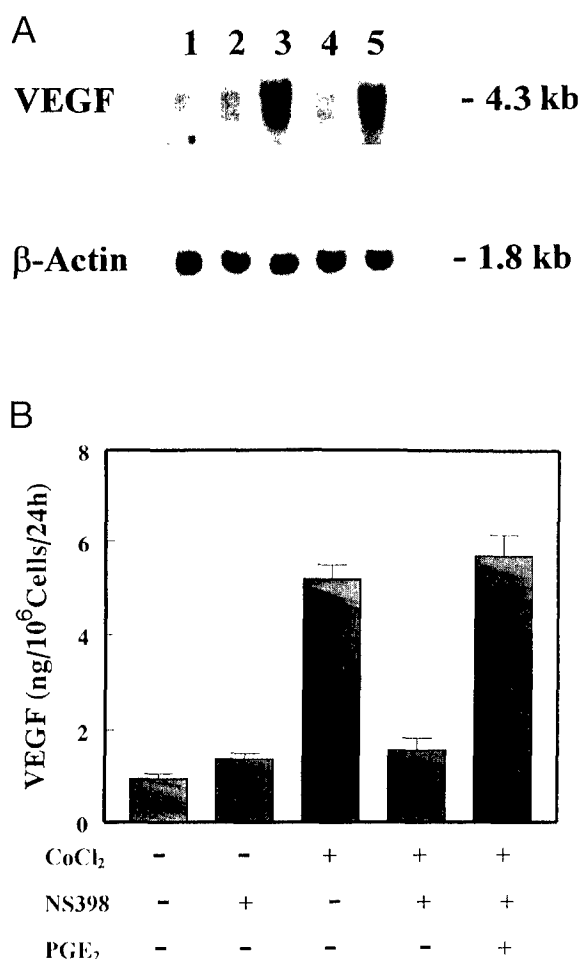


Figure 6. PGE₂ restores the CoCl₂-upregulation of VEGF in the presence of NS398. (A) PGE₂ restores the CoCl₂-upregulation of VEGF mRNA in the presence of NS398. PC-3ML cells were cultured in serum-free medium for 24 h. Incubations were continued with various treatments for 2 d. Lane 1, vehicle as control; lane 2, NS398; lane 3, CoCl₂; lane 4, CoCl₂ + NS398; lane 5, CoCl₂ + NS398 + PGE₂. The concentrations of compounds added were CoCl₂, 100 μ M; NS398, 100 μ M; PGE₂, 10 μ M with daily addition. Total RNA was then extracted and subjected to Northern blot analysis with a VEGF cDNA probe, followed by rehybridization with a β -actin probe. A representative result from duplicate experiments is shown. (B) PGE₂ restores the CoCl₂-upregulation of VEGF protein secretion in the presence of NS398. PC-3ML cells were cultured in serum-free medium for 24 h. Lanes are labeled to correspond with panel A. Culture medium was collected, clarified, and subjected to ELISA to quantitate VEGF secretion. The results were normalized to cell number. The data represent mean \pm SEM from three separate determinations.

Discussion

Overexpression of COX-2 has been linked to an enhanced VEGF expression [11], and increased metastatic potential [10]. However, the underlying mechanism of action of COX-2 is largely unknown. Cellular responses to either hypoxia or cobalt share a common mechanism for oxygen sensing, signal transduction, and transcriptional regulation [2, 21]. The present study created an *in vitro* hypoxia-like state by treatment of prostate cancer cells with 100 μ M CoCl₂ and demonstrates that the biological aggressive, highly invasive and bone-targeting metastatic PC-3ML cell line [22] not only expresses high constitutive levels of COX-2 but also exhibits a further and prolonged elevation in the activity of

this isoform in the presence of CoCl₂. In addition, PC-3ML cell line, despite its low basal VEGF level, is distinguished from the other human prostate cancer cell lines by its ability to dramatically upregulate VEGF production in response to CoCl₂-simulated hypoxia. In contrast, CoCl₂ only induces a transient increase in COX-2 expression and a modest upregulation in VEGF production in PC-3 cells, a less invasive and weakly metastatic prostate cancer cell line [20, 22]. Moreover, CoCl₂ has no effect on COX-2 expression and a limited influence on VEGF induction in non-invasive LNCaP cells [25]. These data demonstrate an association of the level of co-induction of COX-2 and VEGF by CoCl₂ with the invasive and metastatic potential of human prostate cancer cell lines. These observations are in agreement with the reports by Shweiki et al. [26] and Damert et al. [27] that the ability to produce VEGF in response to hypoxia is associated with the aggressive and metastatic behavior in human brain tumors.

Highly regulated COX-2 expression and elevated PGE₂ production have been shown to result in increased expression of genes that normally are induced only transiently [28], and are considered to be a feature of the aggressive, metastatic phenotype of human breast cancer [9, 29]. Our results suggest that hypoxia-induced VEGF induction is maintained by the concomitant, sustained elevation of COX-2 expression and PGE₂ production in the aggressive and metastatic PC-3ML cells suggesting an important role for COX-2 in maintenance of high level of VEGF in tumors under hypoxic condition which may lead to tumor cell invasion and metastasis.

Our data demonstrate, for the first time, that inhibition of COX-2 activity significantly suppresses inducible VEGF production by CoCl₂-simulated hypoxia in PC-3ML cells. These results implicate COX-2 as a potential mediator of hypoxia-induced VEGF expression. Although both VEGF and COX-2 gene expression are known to be regulated by hypoxia in several different cell lines [12, 30], the molecular mechanisms underlying these effects appear to be distinct. Hypoxia-induced upregulation of VEGF expression is mediated by a transcription factor, HIF-1, which binds to *cis*-acting DNA elements containing essential HIF-1 binding sites and triggers VEGF transcription [30]. However, recent reports have indicated that HIF-1 does not appear to be sufficient for the full induction of VEGF in response to hypoxia. It requires unknown cellular factor(s) other than HIF-1 to reach full responsiveness to hypoxia [4, 5, 21]. In addition, the details regarding intracellular interaction between HIF-1 and COX-2 (or PGE₂) need to be elucidated. HIF-1 does not seem to be a regulator of transcriptional activation of COX-2 [31, 32]. However, while COX-2 itself is not a transcription factor [32], it produces PGs within nucleus [32] which could presumably regulate transcription or other nuclear events.

Our observation that exogenous PGE₂ can overcome the suppressive effect of a COX-2 inhibitor on upregulation of VEGF by CoCl₂ indicates an important role for PGE₂ in this process. In support of this observation, PGE₂ has been shown to induce VEGF mRNA expression in osteoblasts [33], synovial fibroblasts [34] and VEGF protein secretion

in PC-3ML cells.¹ We speculated that PGE₂ is involved in the interactive complex of HIF-1 or other nuclear event(s) that mediates the hypoxic induction of VEGF by serving as a co-factor or regulating the activity of other nuclear component, such as activated-protein-1 (AP-1). In support of this hypothesis, a recent report demonstrated that deletion of AP-1 binding sites on VEGF gene resulted in a dramatic reduction of VEGF gene induction by 3-fold in response to hypoxia in C6 glioma cells [5].

NS398 has been reported to be a highly selective COX-2 inhibitor with only a very weak activity against COX-1 [35]. Several *in vivo* studies have demonstrated that NS398, as well as other selective COX-2 inhibitors, are potent anti-inflammatory drugs with very low toxicity [36, 37]. Information derived from the present studies together with our previous findings that inhibition of COX-2 activity by the administration of NS398 selectively induces apoptosis in human prostate cancer cells [38] provides a rationale for the use of COX-2 inhibitors as both anti-tumor and anti-angiogenic agents for the treatment of advanced prostate cancer.

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Note

¹Liu XH, Kirschenbaum A, Levine AC, unpublished observations.

References

- Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990; 82: 4–6.
- Bunn HF, Poyton RO. Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev* 1996; 76: 839–85.
- Wang GL, Semenza GL. Characterization of hypoxia-inducible factor-1 and regulation of DNA binding activity by hypoxia. *J Biol Chem* 1993; 268: 21513–8.
- Guillemin K, Krasnow MA. The hypoxic response: HIFing and HIFing. *Cell* 1997; 89: 9–12.
- Damert A, Ikeda E, Risau W. Activator-protein-1 binding potentials the hypoxia-inducible factor-1 mediated hypoxia-induced transcriptional activation of vascular endothelial growth factor expression in C6 glioma. *Biochem J* 1997; 327: 419–23.
- Taketo MM. Cyclooxygenase-2 inhibitor in tumorigenesis (Part II). *J Natl Cancer Inst* 1998; 90: 1609–20.
- Tjandrawinata RR, Dahiya R, Hughes-Fulford M. Induction of cyclooxygenase-2 mRNA by prostaglandin E₂ in human prostatic carcinoma cells. *Br J Cancer* 1997; 75: 1111–8.
- Tsuji M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase-2. *Cell* 1995; 83: 493–501.
- Liu XH, Rose DP. Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines. *Cancer Res* 1996; 56: 5125–7.
- Tsuji M, Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci USA* 1997; 94: 3336–40.
- Tsuji M, Kawano S, Tsuji S, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998; 93: 705–16.
- Schmedtji JF Jr., Ji YS, Liu WL et al. Hypoxia induces cyclooxygenase-2 via the NF- κ B p65 transcription factor in human vascular endothelial cells. *J Biol Chem* 1997; 272: 601–8.
- Form DM, Auerbach R. Prostaglandin E₂ and angiogenesis. *Proc Soc Exp Biol Med* 1983; 172: 214–8.
- Brawer MK, Deering RE, Brown M et al. Predictors of pathologic stage in prostatic carcinomas: The role of neovascularity. *Cancer* 1994; 73: 678–87.
- Weidner N, Carroll PR, Flax J et al. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993; 143: 401–9.
- Lissbrant IF, Stattin P, Damber JE, Bergh A. Vascular density is a predictor of cancer-specific survival in prostatic carcinoma. *Prostate* 1997; 33: 38–45.
- Toi M, Hoshina S, Takayanagi T, Tominaga T. Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. *Jpn J Cancer Res* 1994; 85: 1045–52.
- Mattern J, Koomagi R, Volm M. Association of vascular endothelial growth factor expression with intratumoral microvessel density and tumor cell proliferation in human epidermoid lung carcinoma. *Br J Cancer* 1996; 73: 931–9.
- Connolly JM, Rose DP. Angiogenesis in two human prostate cancer cell lines with differing metastatic potential when growing as solid tumors in nude mice. *J Urol* 1998; 160: 932–6.
- Connolly JM, Rose DP. A comparison of the expression of the malignant phenotype in two androgen-independent human prostate cancer cell lines after orthotopic implantation in nude mice. *Int J Oncol* 1997; 11: 771–6.
- Goldberg MA, Schneider TJ. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J Biol Chem* 1994; 269: 4355–9.
- Wang M, Stearns ME. Isolation and characterization of PC-3 human prostate tumor sublines which preferentially metastasize to select organs in SCID mice. *Differentiation* 1991; 48: 115–25.
- Levine AC, Liu XH, Greenberg PD et al. Androgens induce the expression of vascular endothelial growth factor in human fetal prostatic fibroblasts. *Endocrinology* 1998; 139: 4672–8.
- Nie DT, Hillman GG, Geddes T et al. Platelet-type 12-lipoxygenase in a human prostate carcinoma stimulates angiogenesis and tumor growth. *Cancer Res* 1998; 58: 4047–51.
- Yang M, Sytkowski AJ. Cloning differentially expressed genes by linker capture subtraction. *Anal Biochem* 1996; 237: 109–14.
- Shweiki D, Itin A, Soffer D, Keshner E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-induced angiogenesis. *Nature (London)* 1992; 359: 843–5.
- Damert A, Machein M, Breier G et al. Upregulation of vascular endothelial growth factor expression in a rat glioma is conferred by two distinct hypoxia-driven mechanisms. *Cancer Res* 1997; 57: 3860–4.
- Han JW, Sadowski H, Young DA, Macara IG. Persistent induction of cyclooxygenase in p60^{src}-transformed 3T3 fibroblasts. *Proc Natl Acad Sci USA* 1990; 87: 3373–7.
- Rolland PH, Martin PM, Jacquemier J et al. Prostaglandins in human breast cancer: evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells. *J Natl Cancer Inst* 1980; 64: 1061–70.
- Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* 1994; 12: 5447–54.
- Appleby SB, Ristimaki A, Neilson K et al. Structure of the human cyclooxygenase-2 gene. *Biochem J* 1994; 302: 723–7.
- Kraemer SA, Arthur KA, Dension MS et al. Regulation of prostaglandin endoperoxide H synthase-2 expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Arch Biochem Biophys* 1996; 330: 319–28.
- Harada S, Nagy JA, Sullivan KA et al. Induction of vascular endothelial growth factor expression by prostaglandin E₂ and E₁ in osteoblasts. *J Clin Invest* 1994; 93: 2490–6.
- Ben-Av P, Crofford LJ, Wilder RL, Hla T. Induction of vascular endothelial growth factor expression in synovial fibroblasts by

- prostaglandin E and interleukin-1: A potential mechanism for inflammatory angiogenesis. *FEBS Lett* 1995; 372: 83–7.
35. Warner TD, Giuliano F, Vojnovic I et al. Nonsteroid drug selectivities for cyclooxygenase-1 rather than cyclooxygenase-2 are associated with human gastrointestinal toxicity: a full *in vitro* analysis. *Proc Natl Acad Sci USA* 1999; 96: 7563–8.
 36. Masferrer JL, Zweifel BS, Manning PT et al. Selective inhibition of inducible cyclooxygenase 2 *in vivo* is antiinflammatory and nonulcerogenic. *Proc Natl Acad Sci USA* 1994; 91: 3228–32.
 37. Futaki N, Yoshikawa K, Hamasaka Y et al. NS398, a novel non-steroidal anti-inflammatory drug with potent analgesic and antipyretic effects, which causes minimal stomach lesions. *Gen Pharmacol* 1993; 24: 105–10.
 38. Liu XH, Yao S, Kirschenbaum A, Levine AC. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. *Cancer Res* 1998; 58: 4245–9.